

## Summer heat fires policy hares

*When the political race to 'cure' the drought problem is over, it will fall to government bureaucrats to sort out a national atmosphere policy*

A FASCINATING example of the decision-making process is now playing itself out in Washington, and anyone who has tried to wield a scientific argument in favour of preferred public policy should pay close attention. For it is undoubtedly the case that congressional attitudes about chlorofluorocarbons (CFCs) and carbon dioxide emissions are influenced far more by the fact that the corn in most of Iowa is nowhere near as high as an elephant's eye, and that outside the House and Senate chambers it has been stiflingly hot, than any number of scientific treatises on the subject. And it is equally true that the need to "do something" will be extremely powerful, at least until the rains come and the memory of the hot summer of 1988 has been cooled by the first blizzard of 1989.

When stories about the greenhouse effect appear on covers of national magazines, the topic gets a publicity boost far beyond what any advertising could provide and for a while at least, there is a positive feedback loop, with each segment of the media seeking to outdo others in covering the story. Then came the drought and heat wave, and editors began to ask, quite legitimately, if the drought was related to the greenhouse effect.

Now the true answer is yes, not in the sense that they are causally related, but merely in the sense that conditions on Earth and in the atmosphere must be having some impact on the weather. But understanding that correlation does not necessarily imply causation is an achievement most—including scientists—find difficult. The strength of politicians in setting policy lies in their ability to work with uncertain information—the way politicians make decisions needed to run the economy and set public policy would never stand up under strict scientific scrutiny, if decisions could wait until a scientific verdict was in.

Senator Tim Wirth (Democrat, Colorado) has proposed legislation that would limit carbon dioxide emissions, map out an energy strategy for the country, protect tropical rain forests, convene an international conference on greenhouse gases and provide new money for atmosphere research. If the need to "do something" becomes sufficiently great, Congress could do far worse than to adopt Wirth's bill and see where it leads.

But it ultimately falls to the career bureaucrats to implement the law, and as a group they are less influenced by magazine cover stories. The Environmental Protection Agency (EPA) has just announced how it will achieve the reductions in CFC use called for in the yet-to-be ratified Montreal Protocol. EPA will limit the production and consumption of CFC-11, -12, -113, -114 and -115 at 1986 levels, and in mid-1993 bring a 20 per cent reduction in these levels, dropping to 50 per cent of 1986 levels by 1998. Production quotas will be allocated to five US companies making CFCs based on their historical market share.

EPA has moved according to the letter of the law. But by moving cautiously to implement the Montreal Protocol, it may find that political events will overtake its new rules. This tortoise and hare relationship between politicians and bureaucrats is probably a good thing, in the long run. The bureaucrats act as a kind of buffer in a potentially volatile solution. Even though the EPA may lag in implementing strict CFC emissions, when it starts to rain, and the temperatures cool, it will fall to EPA to

maintain momentum for reduced emissions for greenhouse gases. Although maddeningly slow and frustratingly complex, the bureaucratic tortoise will reassuringly plod on while the political hares chase after the next magazine cover. □

## More missed chances

*President Ronald Reagan leaves tough decisions on AIDS to his successors*

SEVEN years into the AIDS epidemic, US President Ronald Reagan appears still not to have resolved his ambivalent attitude towards AIDS, nor to have found any way to balance conservative and liberal opinion other than by inaction. Last week he failed to implement in full the recommendations of his own hand-picked commission on the human immunodeficiency virus (HIV) epidemic. At issue was his apparent unwillingness to give his unqualified backing to the notion that those carrying the virus should be protected from discrimination.

Instead of following the commission's advice and issuing an executive order, which would have mandated federal agencies to adopt rules prohibiting discrimination against AIDS-virus carriers in the workplace, or proposing anti-discrimination legislation that would have had still wider effect, the president opted for a weaker course of action. His ten-point plan directs federal agencies to adopt anti-bias guidelines, worked out by the Office of Personnel Management, and only "requests" that businesses and schools "consider adopting" the guidelines.

The reasoning behind the commission's call for vigorous action to back anti-bias recommendations is simple. While compassion dictates that discrimination should not be added to the burden borne by those who find themselves infected with the AIDS virus, common sense teaches that the AIDS epidemic cannot be countered effectively unless all those who may carry the virus can come forward for diagnosis without fear of penalty. The unwillingness of the administration to get down to the practical reality of dealing with the AIDS epidemic has already been amply demonstrated in its endless commissioning of new reports and years of vacillation over a public campaign. It is no coincidence that the ten-point plan also fails to give the president's backing to the commission's suggestion for a massive increase in expenditure on treatment for intravenous-drug abusers, at the highest risk of contracting AIDS.

With 1.5 million Americans now carrying the AIDS virus, the next president will have little time to put off firmer action. Both candidates have said they will back an anti-discrimination law and pledged support for the presidential AIDS commission's proposals. But whether they will be successful in the larger task of effective help for drug abusers depends on how effectively they counter the view, which has dominated President Reagan's thinking, that nothing should be done that appears to reward the behaviours that led people to contract AIDS. The new president will have to accept that effective action requires people to be dealt with as they really are, and not as conservative opinion would wish them to be. □



# New programme for neural network computers?

- DARPA report puts aside hype
- New hardware for brain-like computers

## Washington

A \$400 million programme to take neural network computers out of the laboratory and into wide-scale, practical use is under consideration by the US Department of Defense. The programme is mapped out in a 586-page report\* commissioned by the Defense Advanced Research Projects Agency (DARPA) and previewed at the Second IEEE International Conference on Neural Networks held in San Diego at the end of last month.

The seven-year programme would establish a neural network office, managed by DARPA, that would fund research within the universities, national laboratories and industry. Equivalent amounts spent by other agencies would be likely to push government expenditure on neural network research to over \$1,000 million over seven years; up from the current total of \$40 million a year.

Jasper Lupo, director of DARPA's Tactical Technical Office, who commissioned the report, compared the creation of neural network computers to the invention of the 'atomic bomb' when speaking before an audience of enthusiasts at the San Diego IEEE conference. But the study itself goes out of its way to repudiate exaggerated claims made for neural network computing.

The study director, Bernard Widrow, of Stanford University, prefaced the report by pointing to the "dark shadow" cast by the "hyperbole and extravagant claims" made for neural network systems.

The actual performance of neural network computers is described as "far from awesome". But the belief remains, as Lupo puts it, that they will provide "the next major advance in computing technology". What are now needed are machines powerful enough to test whether building computers inspired by biological nervous systems can provide the human-like abilities that have proved so hard to model in conventional computers.

A neural network computer consists of many simple processors, fully or sparsely connected, whose function is determined by the strength and pattern of their connections. Each simple processor is, in essence, a neuron, and each of its inputs a single synapse, whose weighted input can change with experience. Unlike a conventional computer, with one central processor carrying out operations in sequence,

neural network computers carry out operation in parallel and are generally designed to be trained, — rather than programmed — until a particular input produces a desired output.

But an enormous disparity in computing power divides even the most sophisticated neural network computers from real nervous systems. Computers cannot match a fly's brain, let alone a human's.

The DARPA report estimates that the human brain contains 100,000 million neurons ( $10^{11}$ ), each having roughly 1,000 dendrites that form 100 million million ( $10^{14}$ ) synapses. In neural network terminology that gives the brain a storage potential of  $10^{14}$  'interconnects', a measure of the number of weighted inputs in the system. And given the rate at which nerves fire (100 Hertz), a human brain has the potential to make 10,000 million million ( $10^{16}$ ) interconnects per second. A fly's brain can manage  $10^7$  interconnects, and  $10^9$  interconnects per second which is still way ahead of the most advanced supercomputer — a Cray XMP 1-2, for example runs at  $2 \times 10^6$  interconnects, and  $50 \times 10^6$  interconnects per second. Of course, a Cray supercomputer has not been designed with neural network programming in mind. Special purpose computers now under development should achieve interconnect values twenty times higher in the near future.

But the DARPA report calls for speeds in the  $10^9$ – $10^{11}$  interconnects per second range within three to five years, and of  $10^{12}$  interconnects per second in six years. To do so requires ambitious technology development; in the shorter range gallium arsenide, charge-coupled device and analog-digital hybrid chips, and in the longer-run, optical technology, which will allow a much higher density of interconnects.

Specific applications considered by DARPA are necessarily military, although the report stresses that they would have to be built on generic applications in vision, speech and robotics. Non-military applications are potentially enormous. And, if the US government and industry is not prepared to back development. There may be others who will. It was not lost on those attending the San Diego IEEE conference that representatives were present from the full range of Japanese electronics and industrial companies as well as from Japanese technical magazines and newspapers. Alun Anderson

## Shuttle test failure

THE 20-second flight readiness test firing on 4 August of the space shuttle Discovery's main engines was cancelled when the countdown reached *t* minus 5 seconds because a valve in one of the shuttle's three main engines apparently failed to close.

Technicians at the Cape Canaveral launch pad replaced the faulty valve and its sensor over the weekend, and the critical test has been rescheduled for this week. J.P.

## Molecular ageing

A EUROPEAN network of collaborative research in the molecular biology of ageing and age-related diseases is to be formed with the aim of reducing costs and increasing the international competitiveness of European research. Collaboration will be initiated at the first meeting of the Eurage Molecular Biology Group, to be held in Crete on 7–10 October. At the meeting, recent developments will be outlined by experts in the field from both in and outside Europe and the exchange of materials will be discussed. C.McG.

## Frog suit croaks

A CALIFORNIA court has dismissed a lawsuit against a southern California high school brought by a student who claimed that a class exercise in frog dissection violated her religious freedom. In dismissing the case, the judge said that to push the issue further would border on the absurd.

The lawsuit, filed in June 1987, gained considerable notoriety for Jennifer Graham, a 16-year-old animal-rights advocate. The publicity over her case inspired legislation that passed the California legislature in March, recommending that teachers offer alternative lessons when possible for students who object to animal dissection.

During a pre-trial hearing, the judge asked Graham if she would object to dissecting a frog that died of "natural causes", and she said no. But Davis said the school will steer clear of that option. "What constitutes natural causes", he said. "Hit by a car? Drowned in a swimming pool? There would probably have to be another court case to determine the definition of that."

M.B.

## Ciba head appointed

DR DEREK Chadwick has been appointed director of the Ciba Foundation, a scientific and educational charity which promotes international cooperation in medical and chemical research. Currently at the University of Liverpool, Chadwick's main fields of interest have been in structural, synthetic and computational organic chemistry. With a commitment to international cooperation in science, he has worked and lectured widely throughout Europe, North America and Africa. He will succeed Dr David Evered. C.McG.

\* DARPA Neural Network Study to be published next month by AFCEA International Press, Fairfax, Virginia.



# Atmosphere may rise to the top of the West German agenda

## Munich

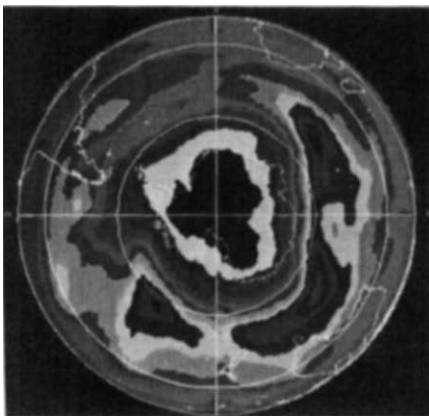
AN impassioned parliamentary report concerning man-made climate change, reduction of stratospheric ozone and rain-forest destruction has shattered the stillness that usually descends on Bonn in summer. Political repercussions are expected when the West German parliament returns in the autumn.

The report, written by Bundestag (parliament) member Bernd Schmidbauer (Christian Democrat), offers a preliminary glimpse of the contents of a forthcoming interim report of the Bundestag's Enquete Commission *Precautions for the protection of the Earth's atmosphere*, of which Schmidbauer is chairman. The commission's full report, to be released in the autumn, will include recommenda-

tions for government action. Sources in the Bundestag expect parliamentary action to follow although the commission will continue meeting until 1990.

In his preliminary report, Schmidbauer calls West German attention to some predictions that the Earth's surface temperature will rise 3–9 °C within a century if the emission of carbon dioxide, chlorofluorocarbons (CFCs) and other 'greenhouse gases' is not reduced.

West Germany is responsible for just 4 per cent (and Western Europe overall 15 per cent) of the worldwide carbon dioxide production of roughly 20,000 million



The springtime ozone hole in the Southern Hemisphere on 5 October 1987. See also the story on the left. (Photo: Scientific American.)

## Lords urge strict measures for CFCs

### London

THE House of Lords has called for measures to halt the destruction of the ozone layer which are more stringent than those of the Montreal Protocol. The Lords Select Committee on the European Communities recommends, in a report published last week, restrictions on production and consumption of chlorofluorocarbons (CFCs) that would reduce emissions of CFCs by 85 per cent. The committee says that the Montreal Protocol, which will halve production, is an "important milestone in environmental policy" and urges member states of the Community to ratify it. But, on the basis of the conclusions of a review group on stratospheric ozone, the committee says the reductions of CFC emissions in line with the protocol will not prevent further depletion of the ozone layer. And it urges that the Community amends it. Even a reduction of 85 per cent would only stabilize chlorine in the stratosphere; if the atmosphere is to recover, then production of CFCs must be phased out completely, it says.

It also demands an immediate ban on the use of CFCs in non-essential aerosols, a step already taken in the United States and other countries. And it calls for an amendment to the protocol to prevent export of aerosols to countries not party to it. At present, this is "a hole in the Protocol as big as the hole in the ozone layer", says the committee's chairman, Lord Cranbrook. The committee recommends that the government reviews its support for research into the ozone layer and says the development of substitutes for CFCs and halons must be speeded up. Christine McGourty

tonnes. The United States produces about 25 per cent and the Soviet Union and the Soviet bloc 22 per cent. Schmidbauer argues that West Germany should lead a drive to try to reduce world emissions.

Schmidbauer, echoing the West German Environment Ministry, calls for stricter international guidelines on CFC release both because CFCs add to the greenhouse effect and because they are implicated in the depletion of stratospheric ozone. Schmidbauer and the Environment Ministry call for a reduction of 85–95 per cent in current levels of CFC production by 2000.

West German industry produced 112,000 tonnes of CFCs in 1986, more than 10 per cent of the world total. Thirty-five countries pledged at Montreal in September 1987 to reduce CFC output by 50 per cent by 1999. Neither the Montreal protocol nor its predecessor, the Vienna protocol of 1987, has been ratified by West Germany, although both are on the parliamentary agenda in the autumn. A ministry spokesman said that persuading industry to accept huge production cuts would be "the least of our problems" compared with achieving an international agreement for further reductions in CFC production beyond the Montreal limits.

## Congress investigates 'six-pack rings'

### Washington

THE plastic 'six-pack ring' found itself the unlikely subject of a hearing by the US House of Representatives Committee on Merchant Marine and Fisheries last week. The ring might seem too humble an item for congressional scrutiny, even though it does hold together an important American institution — the pack of six cans of drink that are vital to the enjoyment of a summer's day at the beach. But to the wildlife experts who testified at the hearing, the ring is more than just an ordinary piece of plastic.

In order to grip the cans firmly by their rims, six-pack rings have to be very strong. And, often enough, the rings end up thrown away into the sea, where they float for years. In the sea they are a hazard: animals eat them and starve, or become entangled in them. Sea-birds may get the rings caught around their necks; thus weighted down they are slow to take flight and become an easy catch for predators.

The answer to these problems under consideration by the congressional committee was S.1986, federal legislation requiring the rings to be made of degradable plastic. Seventeen states, most of them coastal, have laws or will soon be implementing laws to prohibit the use of non-degradable plastic rings and other carriers.

Al Manville, senior staff wildlife biologist for the Defenders of Wildlife, pressed for the legislation, saying that the United States could not afford to wait any longer to start doing something about the problem of plastic materials.

The Environmental Protection Agency, Illinois Tool Works and the Society of the Plastics Industry agreed that more research is needed, but opposed the passage of a bill until the best solution could be found. The United States Fish and Wildlife Service does not support the bill. Elizabeth Ebbert

West German Chancellor Helmut Kohl (CDU) has taken a personal interest in slowing the destruction of the world's tropical rainforests using debt relief for the developing countries involved. He and Environment Minister Klaus Töpfer (CDU) will press for international co-operation at the World Bank meeting in West Berlin in the autumn.

West Germany faces a number of problems in reducing its contribution to the greenhouse effect. Its only indigenous energy resource, coal, is the worst fossil fuel in terms of carbon dioxide release. Natural gas, the cleanest-burning fuel, is not found in West Germany, and nuclear power has not been a popular option, especially since the Chernobyl incident. The experts called before the Enquete Commission recommended energy conservation as the only practical way to reduce emissions. Steven Dickman



# Reagan's U-turn on acid rain seen as election strategy

## Washington

IN a politically motivated policy reversal, President Ronald Reagan has apparently agreed that the United States should freeze nitrogen-oxide emissions at 1987 levels, as proposed in international negotiations on a treaty designed to reduce acid rain. Word that Reagan supported the decision to adopt the freeze, reached at a meeting of Cabinet members involved in domestic policy, emerged at the end of last week.

# Gorbachev rallies the Soviet academy

## London

THE leadership of the Soviet Academy of Sciences must reconsider its programmes for the development of fundamental research, Mr Gorbachev told the Central Committee of the Communist Party of the Soviet Union, during a plenary meeting on implementing the resolutions of the recent party conference. *Perestroika* (restructuring), Gorbachev said, is impossible without the "active assistance of science", and, the state bodies — in particular the USSR State Planning Committee and State Committee for Science and Technology — must therefore examine ways to improve radically the financial and equipment base of science. Structural and investment policy, he said, must be revised to ensure the creation of favourable conditions for the development of priority trends.

Many major scientific centres, Gorbachev continued, are satisfied to play the part of commentators and critics, and have not noticed that *perestroika* has advanced from the "rally stage" to the "profound transformation" of all spheres of Soviet life. The criticism was directed at social scientists, but will have been noted by those in other fields. Somewhat surprisingly, in his address to the central committee, Gorbachev made no reference to the "scientific-production associations" which three years ago he advocated as a means to cutting through bureaucratic barriers and bringing science closer to production.

In spite of Gorbachev's urging, relatively few such associations have been established, and even fewer have proved successful. The latter, in fact, have turned out to be little more than a new label for the long-standing involvement of particular research institutes (such as the Paton Welding Institute of the Ukrainian Academy of Sciences) with local industry. The rest, as far as can be judged from the somewhat circumspect comments of the scientists involved, have not so much broken through the bureaucracy but simply added another layer to it.

Vera Rich

The United States is one of the biggest contributors to global levels of nitrogen oxides, but the Reagan administration has been reluctant to acknowledge the role of nitrogen oxides and sulphur dioxides in acid rain, and to yield to international pressures to institute emission controls. The United States abstained from signing a protocol to limit sulphur-dioxide emissions which was agreed to by 16 nations including the Soviet Union in 1985, and the US failure to limit emissions linked to acid rain in Canada has been a major sticking point in relations between the United States and Canada. Reagan's agreement to the freeze on nitrogen-oxide emissions is being seen as an election-year attempt to bolster the Republican stance on environmental issues.

The current protocol stems from a United Nations-sponsored meeting held in Geneva in April of the signatories to the 1979 Convention on long-range transboundary air pollution. The convention, sponsored by the Economic Commission for Europe, involved 34 nations which agreed "gradually to reduce and prevent air pollution, including long-range transboundary air pollution".

Discussions at the April meeting boiled down to a dispute between the United States and Canada over the "transboundary flow" of nitrogen oxides which are produced in the United States and blow over the Canadian border. A clause specifically restricting transboundary flows to 1987 levels was introduced by the Canadians to counter an amendment submitted by the United States that would set the freeze levels at those of 1987 or "any previous year."

Because US emissions generally have been declining over the past ten years, US negotiations sought some "credit" for past cutbacks while retaining some breathing room should emissions rise. US officials have repeatedly said that it is impossible to determine what proportion of domestic emissions are transported across the border, so by setting transboundary flows at 1987 levels, the Canadians effectively forced the United States into freezing emissions at those levels.

Canada has already frozen its nitrogen oxide emissions, but nitrogen oxides — primarily produced by automobile emissions — are only a part of the acid rain story. Sulphur dioxide, almost all of it produced by coal-fired electricity generating stations, represents a far more intractable problem. The Reagan administration has put its faith into clean-coal technologies which will not mature until the next century.

The United States and Canada are

# SERC support for materials research

## London

THE Science and Engineering Research Council (SERC) last week set up a materials commission to coordinate research and improve links with industry. But the chairman of the commission, Professor Colin Humphreys of the University of Liverpool, says that an increase in funding for materials research is vital if Britain is to keep up with the world leaders in this field.

Humphreys says that funding has not increased in recent years, except in superconductivity research. And as other countries, particularly Japan and the United States, intensify their efforts, Britain is "in grave danger of falling behind". The commission takes on the role and funds previously assigned to the engineering and science boards and will administer a grants budget of £15 million. An additional £2.7 million from SERC will go towards setting up a new interdisciplinary research centre.

Professor Bill Mitchell, chairman of SERC, says that the council's support of materials research has been very fragmented and the new interdisciplinary commission will lead to a more integrated approach. It will have responsibility for research from synthesis of materials to prototype devices and demonstrator projects, and will respond to both "market pull and science push", says Humphreys.

The commission will comprise four new committees: in ceramics and inorganic materials; polymers and polymer composites; metals and metallurgy; and semiconductors, plus three existing committees for molecular electronics, superconductivity and medical engineering. On each committee there will be both academics and industrialists. Assessors from government departments will sit on the commission as a first step towards forming national materials-research priorities.

This new initiative indicates the high priority SERC is giving to materials research. "Materials underpins a wide range of wealth-creating technologies", says Mitchell. Some new materials on the horizon are sieves which are specific to particular molecules, ceramic materials which are superconducting at room temperatures and can carry critical currents and alloys with shape memories. Christine McGourty

expected to work out their differences before 31 October, which is the date the treaty will be signed in Sofia, Bulgaria. The treaty enters into force 90 days after it has been signed and ratified by all parties involved, and it stipulates that six months later negotiations should begin on "real reductions" to reduce emissions of nitrogen oxides below the freeze levels, which must be accomplished no later than 1996. □

# Proposed AIDS screening causes political turmoil in France

Paris

AN ill-timed proposal for systematic screening for the AIDS virus in pregnant women and patients due to undergo surgery was at the heart of a controversy that rocked the new French government last month. As the newly elected socialists struggle with a hung parliament, this affair shows not only that AIDS is a politically sensitive issue, but that the government's strategy for opening access to power for non-career politicians may not be the way to get the support it needs to govern.

Just nine days after he appointed his new government — for the second time in under two months — Prime Minister Michel Rocard found himself forced to demand the resignation of two of his junior ministers, both of them new to politics. One of these, Leon Schwartzberg, a practising haematologist who sees himself as a crusader for patients' rights, found that his first lesson in political protocol, the need to consult his peers, will benefit his successor more than himself.

Schwartzberg was given the health portfolio under Claude Evin, minister for solidarity, health and social security. At his first press conference on 5 July, Schwartzberg surprised and embarrassed the government by unveiling plans for systematic screening for AIDS, criminal

proceedings against employers who discriminate against seropositive employees, the introduction of measures to combat drug addiction and an obligation for doctors to give patients a copy of their medical records after each treatment. These plans had not been discussed with Evin nor with medical colleagues.

In an attempt to restore decorum, Evin immediately announced that Schwartzberg's proposals would be put before the national ethics committee, as well as two national medical committees, before being taken further. Within 24 hours it was clear that Schwartzberg did not have the support of his peers. An epidemiological study of AIDS in pregnant women had already suggested that systematic national screening for the virus was not yet justified and could threaten human rights. It also emerged that patients have had a statutory right to see their medical records since 1970.

By 7 July, 48 hours after his first press conference, Schwartzberg had resigned. The prime minister tried to make light of the affair, saying that he respected Schwartzberg's humanitarianism, but felt that political coherence has to be maintained, but other cabinet members were saying that *faux pas* could be the price paid for the government's 'demo-

cratic' will to open government posts to non-politicians. Members of the opposition were less kind, speaking of "ineptitude" and "government through the media."

Schwartzberg's return to his post at the Paul-Brousse hospital on the outskirts of Paris may mark the end of the shortest-ever career of a French politician.

Peter Coles

## Nuclear waste policy attacked

London

BRITAIN'S nuclear waste policy has come under severe criticism from the advisers to the government, the Radioactive Waste Management Advisory Committee. In its recent annual report, the committee criticizes aspects of national policy as "confused" and "deficient". And it says that some dumps are not properly monitored because of a lack of resources.

The committee warns that there is "enormous uncertainty" whether a deep disposal site for low and intermediate level waste will be found and in use before the year 2005 as planned, though the disposal site now used for low level waste, at Drigg near Sellafield, in the north of England may be full by 1999. The chairman of the committee, Professor John Knill of Imperial College, says that British Nuclear Fuels Limited must make more efficient use of the space available or apply for an extension of the disposal area, entailing a planning inquiry.

Intermediate-level waste is presently in storage pending a decision on an acceptable disposal method. In the meantime there is confusion, says Knill, on how to encapsulate the waste as that is dependent to some extent on the disposal route.

Another item on which policy remains confused is how to dispose of large-scale items from decommissioned nuclear reactors. No decision has yet been taken on whether long-term storage, deep disposal inland or disposal at sea is an appropriate method. This question will become of "increasing urgency" in the next decade, says Knill, "and is just one of many which needs resolution".

Monitoring of dumps for very low-level waste also comes under criticism in the report. The body in charge of administrative and technical controls, Her Majesty's Inspectorate of Pollution, is understaffed, with a heavy workload which may increase in the future, says Knill. As a result, there is no structured programme of monitoring after initial site assessment and sites are not monitored or recorded after closure. This is necessary to protect the sites from accidental intrusion or inappropriate future use, says the report.

Christine McGourty

## Companies to pay in toxic clean-up accord

Boston

THE US Environmental Protection Agency (EPA) announced last week the largest single cash settlement from the highest number of companies in the history of the federal toxic waste cleanup programme called Superfund. In the agreement, more than 350 companies will pay a total of \$49 million — \$17 million of it in cash payments to EPA — to clean up four sites in Massachusetts and New Hampshire where a private disposal company illegally dumped toxic waste.

Years of midnight dumping by the Cannons Engineering Corporation left a legacy of more than 180,000 cubic feet of soil contaminated with hundreds of thousands of gallons of liquid waste oils, solvents and other toxins. In the EPA settlement, area companies using the Cannons waste management company will have to pay the bulk of the clean-up because the Superfund law makes waste generators responsible for their toxic waste from 'cradle to grave'. The settlement awaits official authorization from a federal judge, and also leaves outstanding a dispute over \$9 million in further costs against 25 companies that have refused to pay for the clean-up.

The list of companies entangled in the clean-up mess includes some of the largest corporations in the United States, such as



General Electric, Monsanto and Atlantic Richfield. The list also includes some of the most highly respected local concerns: the Archdiocese of Boston, the *Boston Globe* newspaper and Polaroid Corporation. Although the waste management company has long since declared bankruptcy, five of the individuals responsible for the actual dumping face gaol sentences.

Seth Shulman



## Japan announces its "special distinguished grants" for 1989

Tokyo

BRAIN-RELATED research features prominently in this year's "special distinguished grants" announced at the end of last month by Japan's Ministry of Education, Science and Culture, taking three out of the ten awards. Big grants also go to research on solar flares and to development of new high-temperature superconductors to be made not from oxides but from organic compounds. The grants are the ministry's most lucrative and are intended for "internationally recognized research likely to produce outstanding results". Awards run for 3–5 years and are typically worth \$1–2 million.

As in previous years, molecular biology dominates the research projects selected. Professor Michio Ui in the Faculty of

Pharmaceutical Sciences of Tokyo University receives ¥279 million (\$2 million) over four years to study the molecular signalling systems that control cell growth and differentiation. And Professor Toru Yoshizawa of Kyoto University receives ¥190 million over three years to investigate the molecular dynamics of colour and dark-light recognition in the eye.

The biggest grant of all, ¥316 million for four years, goes to Professor Shosaku Numa of Kyoto University to continue his internationally recognized research on neurotransmitter receptors and ionic channels in muscles and the brain, Numa's second special distinguished grant.

With his new grant, Numa intends to elucidate the structure and function of the nicotinic acetylcholine receptor and sodium channels at the molecular level, to locate voltage sensors in the sodium channel, and look at the physiological characteristics of calcium channels and the muscarinic acetylcholine receptor.

Spiders will feature prominently in a three-year study of the brain by Dr Nobufumi Kawai of Tokyo Metropolitan Institute of Neurosciences, for which he receives ¥181 million. The Japanese silk spider (*Nephila clavata*) injects insects trapped in its web with a poison that latches onto glutamine receptors in the insect's muscles, thereby paralyzing the victim. In higher-level organisms, such as man, glutamine receptors are found in the brain and Kawai intends to locate the receptors using spiders' toxin labelled with isotopes. He also hopes to make glutamine receptor antibodies and use animal models of neural disorders to elucidate brain diseases.

Professor Hiroshi Wada in the medical faculty of Osaka University, on the other hand, will use more conventional techniques in his study of histamine receptors in the brain, for which he receives ¥174 million over four years.

In the physical sciences, Dr Katsuo Tanaka of the National Astronomical Observatory will observe solar flares during the next solar maximum in 1991 with the X-ray satellite Solar A, which is due to be launched that year by the Institute of Space and Astronautical Science. And with his ¥216 million grant his team will also develop a land-based telescope equipped with four sensors to observe sunspots, magnetic fields that connect pairs of sunspots, hydrogen alpha-radiation emitted from the periphery of flares created in the magnetic field, and gas flow on the solar surface.

As last year, a big grant goes to the development of new superconductors. But, interestingly, the award this year

## Devil-may-care policies attacked

Paris

THE French Secretary of State for the Environment, Brice Lalonde, is under attack from both sides of the globe, accused of supporting a 'devil-may-care' attitude towards environmental pollution. For a former president of the French section of the ecology action group, Friends of the Earth, this criticism is hard to swallow.

The controversy sprang up around a decision taken in Luxembourg on 28–29 June by environment ministers of the European Economic Community (EEC) to apply strict norms to limit pollution from car exhausts throughout the member states. Having accepted, reluctantly, the majority decision that catalytic converters should be fitted to small as well as larger cars, Lalonde changed his mind.

The problem, ostensibly, is that while the EEC has agreed minimum standards, some countries — notably West Germany and the Netherlands — have decided, unilaterally, to apply even stricter controls, in line with those already in force in the United States. This, said Lalonde in a statement issued on 20 July, makes an ass of the interministerial meeting, which, after all, sought to apply common norms.

But there may be a more subtle motive behind Lalonde's *volte face*. Even before the June meeting in Luxembourg, Jacques Calvet, the director of Peugeot SA, one of France's two main car manufacturers, made it known that he disapproved of the proposal to require catalytic converters to be fitted to small cars. This, he said, would substantially increase production costs, reducing the international competitiveness of cars whose principal selling-point is their low cost. West Germany, he argued, would be less affected, as it mostly manufactures bigger cars, at the top end of the market, where the price increase would be less noticeable. Although France's other chief car manufacturer, Renault, has not voiced the same objections, it is thought that Calvet's remarks influenced the French government's decision.

Peter Coles

## West Germany calls for ocean clean-up

Munich

WEST German Environment Minister Klaus Töpfer (Christian Democrat) proposed a DM20,000 million plan last week for cleaning up the North Sea. Increased waste-disposal fees of between DM140 and DM300 annually per household would pay for the removal of nitrates and phosphates from sewage, costing DM14,000 million overall. Such pollution has been widely identified as a possible cause of the sudden death of more than 2,000 seals in the North Sea this summer.

In addition, industry would be called upon to contribute "several thousand million" Deutschmarks for various clean-up projects, said an Environment Ministry spokesman. The dumping of dilute sulphuric acid into the North Sea would be forbidden from 1989, and the burning of toxic waste on the high seas, starting in 1994.

Töpfer also called upon other countries to join in the anti-pollution crusade. The Scandinavian countries and the Netherlands have already declared their willingness to help. He specifically invited East Germany, Switzerland and Czechoslovakia to participate in the next North Sea conference in 1990. Until now, these countries have refused to participate.

The opposition Social Democrats (SPD) claimed that Töpfer's programme is insufficient; they called it unfair to charge citizens more for sewage treatment while industry is handled "with kid gloves". The SPD proposed investing DM40,000 million to clean up the North Sea, with the lion's share to be paid by industry.

Steven Dickman

is to develop "high-temperature organic superconductors". The first organic superconductor was discovered in 1980 and since then about 30 superconducting organic compounds have been found, many of them by Japanese scientists. But the temperature at which they become superconducting is far below that of the new oxide superconductors. Nevertheless, Professor Isao Ikemoto of Tokyo Metropolitan University is determined to "challenge" the oxide superconductors with his ¥254 million grant.

David Swinbanks



# Big is beautiful for survival of higher education in Australia

*Sydney*

**FORCED** amalgamations for tertiary institutions with fewer than 2,000 students is the most contentious element of new Australian government policy on higher education announced at the end of last month by Mr John Dawkins, Minister for Education, Employment and Training. The policy gives effect to most of the reforms suggested last December (see *Nature* 330, 592; 1987).

The central element of the policy is the establishment of a 'unified national

general recurrent, equipment, minor works and special research grants.

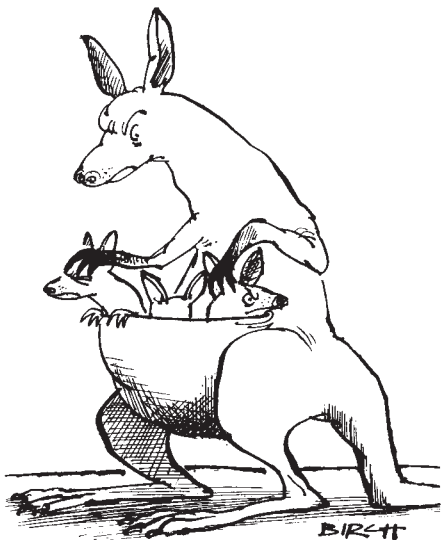
Institutions choosing not to join the unified system will be funded for teaching only, receiving nothing for growth or research. Institutions with fewer than 2,000 students, of whom there are at present 21, will not be allowed to join the unified national system. Pressure will also be put on institutions with between 2,000 and 5,000 students to amalgamate. The minimum number of students is 5,000, at which the government believes an institution will be able to deliver a comprehensive range of courses as well as being able effectively to involve itself in research in

some specialized areas.

Private institutions such as the Bond University will not receive any government support, nor will their students be eligible for study benefits. Staff will, however, be able to compete for research grants from the Australian Research Council (ARC). Money for research will be transferred from operating grants to the ARC in stages, until the ARC has an extra A\$65 million in 1991.

The policy statement emphasizes the government's desire to improve management practices in higher education. Dawkins says that it is impossible for some university senates and college councils with up to 50 members to operate efficiently. He says a more appropriate size would be 10–15, in line with the size of boards in large private organizations.

**Charles Morgan**



system' for higher education. In order to join, an institution will have to prepare an 'institutional profile' which sets out its objectives and strategy for teaching and research as well as how it is addressing national priorities as defined by the government. Funding will be based on the institutional profile as well as objective statistical measures of performance compiled by the government. From 1989, funding for institutions in the unified national system will be a single operating grant which will replace present separate

## UK universities turning more and more to private funding

*London*

**UNIVERSITIES** in Britain are relying increasingly on private rather than public funding. Statistics published this month by the Universities Statistical Record, which detail income and expenditure in 1986–87, show that the proportion of university funding from central government grants fell to 55 per cent, compared with 57 per cent and 59 per cent in the previous 2 years and 75 per cent 10 years ago.

Last year's grant of £1,400 million was a 4 per cent increase on the previous year. And the total income of universities increased by 8 per cent to almost £2,500 million. But the underlying trend is an increase in the proportion of university income earned from research contracts and services, a trend that the government is encouraging. Last year, the funding generated in this way rose by 18 per cent, almost £100 million, to £630 million.

There was an increase in funding of 28 per cent from charities, of 22 per cent from overseas organizations, of 16 per cent

from British industry and of 14 per cent from the research councils.

Income from fees also rose, due largely to the increase in fees charged to overseas students. Fee income rose by nine per cent from the previous year, £26 million to £330 million, the same level as five years ago; since then income from full-time students paying home fees has fallen by almost one-third.

Universities' expenditure increased by 6 per cent to £1,850 million, bringing the increase over the past five years to almost one third. Last year, 68 per cent of general funds went on salaries and wages, an increase of 7 per cent on the previous year, and of almost one-third over the past five years. The largest rise (54 per cent) was for academic-related staff such as administrators and computer staff, compared with 20 per cent for technical staff.

Over the past five years, student-staff ratios have increased in most departments, particularly in clinical subjects, because reductions in full-time staff have been substantially greater, says the report, than the changes in student numbers. Last year there were about 55,000 full-time staff and 3,500 part-time academic and related staff, a 3 per cent rise over the previous year. That increase was due to an increase of 9 per cent in the number of staff not paid from university funds. Those staff now constitute one-third of all full-time academic staff, compared with 23 per cent 5 years ago. There was no increase in the number of full-time teaching and research staff paid from general funds. Of staff not paid from general funds, 38 per cent are financed by the research councils, 21 per cent by other government departments and 10 per cent by industry.

**Christine McGourty**

## Spanish Cabinet in science reshuffle

*Barcelona*

**IN** a cabinet reshuffle, Mr José María Maravall has been replaced as Spanish Minister of Education and Science by Mr Javier Solana, former Minister of Culture, who will take over at a delicate time for education and science in Spain.

Maravall, Minister of Education and Science since the first socialist government in 1982, has been responsible for important reforms in primary and secondary schools, in universities (the Law for University Reform) and in science (the Law of Science and the National Plan for Research).

Solana, a physicist with experience in

research and university, is considered a personal friend of Prime Minister Felipe González and a person open to dialogue and negotiation. He takes charge at a time when new action on salaries is expected in September at all levels of education, including the universities, and when criticisms are increasing on the way the Law of Universities is working. He will have to continue the efforts to put into practice the National Plan of Research, now in its first year, and to implement the new statutes of the CSIC (Science Research Council) which have to be approved soon.

**Pedro Puigdoménech**

# South Africa continues to put off signing nuclear treaty

*Oxford*

SOUTH Africa again faces possible expulsion from the International Atomic Energy Agency (IAEA) at its annual general assembly in September. This follows its continued refusal to sign the Nuclear Non-proliferation Treaty (NPT), despite a statement by State President P.W. Botha on the eve of last year's general assembly that it was prepared to begin negotiations to that end. He added that South Africa would consider accepting safeguards on all its nuclear installations (a specific provision of the treaty), but that Pretoria's attitude would depend on the outcome of the assembly.

Botha's statement successfully stalled a Nigerian bid to expel South Africa, following a recommendation from the agency's board of governors last June that South Africa be suspended. This in turn followed the breaking off of IAEA negotiations with South Africa in December 1986 over safeguards at its new commercial enrichment plant, and 16 years of unsuccessful attempts to get it to sign the NPT. Interestingly, the United States secured the cooperation of the Soviet Union in thwarting South Africa's expulsion, on the grounds that the agency was able to exercise more control over its nuclear programme if it remained a member. A compromise solution was instead adopted, agreeing to renew attempts to expel South Africa in a year's time, giving it a period of grace in which to realize its stated intentions. Proposed by the Mexican delegation and seconded by Algeria, this resolution was adopted by 60 votes to 28, the United States, the European Communities and other Western countries voting against it.

Botha raised Western hopes that South Africa would sign the treaty at last; Richard Kennedy, deputy head of the US delegation and a member of the agency's board of governors, criticized the failure of the resolution to take note of "the potential substantial development" posed by Botha's statement. This refers to the fact that South Africa had never before publicly countenanced acceding to the NPT. These hopes appear to have been ill-founded, however, and if voting follows a similar pattern this year, the necessary two-thirds majority required to ensure South Africa's expulsion is likely to be narrowly achieved.

South Africa may well try to stave off expulsion yet again by offering to place under IAEA safeguards either its commercial enrichment plant or possibly both it and its pilot enrichment plant, while still refusing to sign the NPT. This would require little sacrifice on South Africa's

part, as it would forfeit only the possibility of sabotaging the non-proliferation regime by exporting highly enriched uranium without IAEA safeguards in the future. As the commercial enrichment plant came on line recently but is unlikely to reach full capacity for some time, the possibility of South Africa becoming an exporter of highly enriched uranium, as it originally intended, seems very slim. Although South Africa will be capable of supplying the two reactors of the country's existing nuclear power station at Koeberg when current stocks of highly enriched uranium run out, it may even have difficulty supplying its planned second nuclear power station.

The pilot enrichment plant is widely regarded as the most likely source of South Africa's nuclear weapons, assuming

they exist. This plant was commissioned in 1975, but became fully operational only in March 1977. Based on its capacity of 6,000 separative work units, it has been calculated that it is capable of producing up to two nuclear weapons each year. It could have produced one or two nuclear weapons by August 1977, when a testing site (subsequently dismantled) was detected by satellite in the Kalahari Desert. South Africa could subsequently have stockpiled about 20 nuclear weapons, and could thus place this plant under safeguards as well, while not undermining the possibility of its nuclear capability.

If South Africa does already possess nuclear weapons, it has little option but not to sign the NPT, and last year's statement was just yet another case of Botha successfully leading the West up the garden path. If it does not, then it will continue to delay signing as long as possible in order to keep the world guessing about its nuclear intentions.

**Michael Cherry**

## Even conservatives share conservationist view of Soviet lake

*London*

Mr Egor Ligachev, the member of the Politburo of the Communist Party of the Soviet Union (CPSU) who is considered by western observers to be the custodian of hard-line conservative ideology, has sharply attacked the progress of conservation work at Lake Baikal. This lake, the largest and deepest body of fresh water in the world and the habitat of several unique species, including freshwater seals, came under increasing environmental threat during the Brezhnev era, in particular from the development of industries around the lake. Mr Gorbachev's *glasnost* campaign, however, brought to light the problems of the lake, and in April 1987 a special government and CPSU Central Committee resolution set out a programme for the protection of the lake and the "rational use" of it.

Although much has been done to implement this programme, Ligachev said, in particular, the closure of the yeast-producing facility at the Baikal pulp and paper combine, the rate of progress is too slow. Several ministries and government bodies are behind schedule in the installation of anti-pollution devices. The special Interdepartmental Monitoring Commission for the natural complex of Lake Baikal is failing to make full use of its statutory powers to deal with backsliding organizations, Ligachev says, and the funds and equipment allotted to pollution control have not always been used effectively. Researchers concerned with the problems of the lake, or working in enterprises situated close to its shores or tribu-

aries, Ligachev says, must make greater efforts to develop and introduce waste-free technologies.

A particular black spot, environmentally speaking, is the north shore of the lake, in particular the new town of Severobai-kalsk, which is being developed without proper study and in an "ill-thought-out manner". The very existence of this town has recently been questioned in the Soviet media. Ligachev appears to share these views asking "is it really necessary to create yet another industrial centre here, at Severobai-kalsk".

Ligachev urges that to be effective the whole package of conservation measures for the lake must be carried out simultaneously and "unconditionally". Yet he did not mention one of the main threats to the well-being of the lake, the condition of the Selenge river. This river which provides about 60 per cent of Baikal's water, rises in the Mongolian People's Republic, where indiscriminate tree-felling has led to a considerable decrease in the Selenge's flow. During the past few months, the Mongolian government and Party have shown an increasing awareness of this problem and the need to embark on urgent re-forestation work, and at the beginning of July, the first ever Soviet-Mongolian environmental round table was convened in the Mongolian capital, Ulan Bator, to discuss the protection of lakes Baikal and Huvsgul. But Ligachev makes no reference to this meeting, nor to the Mongolian aspect of the Baikal problem, but treated it as if it was a matter purely for the Soviets.

**Vera Rich**



# US/Soviet proposal to ban nuclear power in Earth orbit

## Berkeley

THE impending fall of Cosmos 1900 (see right) promises to draw attention to a joint proposal by US and Soviet scientists to ban nuclear power in Earth orbit.

The plan, proposed by the Federation of American Scientists (FAS) and the Committee of Soviet Scientists Against the Nuclear Threat, led by Roald Sagdeev, soon to leave his post as director of the Soviet Space Research Institute, would serve two purposes: to end the environmental hazard of radioactivity raining down from orbit; and to curtail an impending arms race in space. Verification of compliance with such a ban would be straightforward, the scientists say, as nuclear reactors can be readily detected with infrared, gamma and neutron radiation. And the ban would not prohibit nuclear powered deep-space exploratory or scientific missions.

The United States has not launched a nuclear-powered spacecraft since the Voyager probes in 1977, but is developing a new generation of nuclear reactors, for use in the Strategic Defense Initiative (SDI) as well as other applications. Although the Reagan administration promised the US public that SDI would be "non-nuclear", there is some confusion over whether that pledge refers only to nuclear weapons or to power as well. Most experts agree that the directed-energy weapons proposed for SDI will not be possible without long-lived, manoeuvrable and radiation-tolerant nuclear reactors, and that orbiting nuclear power stations are necessary for most military applications in space.

If Cosmos 1900 had to fall, it picked a fortuitous time, says Steven Aftergood, director of the Committee to Bridge the Gap, a Los Angeles-based public interest research organization opposed to nuclear space technology. "It will provide a useful illustration of at least some of the hazards

involved", he said. But environmental concerns alone are not enough to justify a ban on orbiting reactors, says Aftergood, who helped to draft the proposed ban.

The re-entry problem could be largely solved if both superpowers agreed to orbit reactors only above a minimum safe altitude. Launch failures are not a hazard with fission reactors as they are with plutonium-powered radioactive thermoelectric generators (RTGs), as uranium itself is not particularly hazardous, and fission is not initiated until the reactors are



in place in orbit. Moreover, the ban, as proposed, would still allow deep-space missions to carry RTGs, whose highly radioactive plutonium does present a launch hazard.

Although a nuclear reactor ban would require the Soviets to redesign their RORSAT system, there is nevertheless optimism among the scientists that Sagdeev will win the support of Soviet officials for the ban. Congress has also shown interest, even though the ban would imply the end of SDI. The plan may be the subject of committee hearings in the next few months. **Marcia Barinaga**

# Rights of patients to immortalized cells

## Washington

A SURPRISE ruling by a Californian court backs the notion that patients have the right to control what happens to samples of tissue and body fluids taken from them, and have a financial interest in any products developed from them.

The case arose after a Seattle businessman, John Moore, filed a suit against the University of California at Los Angeles (UCLA) Medical Center where he had been successfully treated for hairy-cell leukaemia. Cells from his spleen, which was removed as a standard part of his treatment,

were found to have unique properties that made them useful as a cell-culture source of interferons and colony-stimulating factor. UCLA researchers established a cell line from the spleen cells which the university then patented and developed in collaboration with two biotechnology companies.

The case may continue for years and eventually be appealed in the Supreme Court of California. In the interim, biotechnology companies will have to prepare themselves for the possibility that they will have to share profits from cell lines with the patients who provided the cells. **Alun Anderson**

# Failed satellite to crash soon

## Berkeley

THE third Soviet nuclear-powered satellite to fall from orbit will re-enter the atmosphere in August or September. The Soviets have lost radio contact with Cosmos 1900, a radar ocean reconnaissance satellite (RORSAT), preventing boost of the satellite to a stable high-altitude orbit, and ensuring that it will return to Earth, as its orbit continues to decay.

The low orbits (around 250 km) of the RORSATs make them vulnerable to re-entry into the atmosphere, and also dependent on nuclear power, as the large surface area of solar panels would create intolerable drag. After the satellites' brief functional life of several months, they are normally boosted to an orbit of close to 1,000 km, where they will remain for at least 300 years, long enough to allow the decay of most of their fission products.

Cosmos 1900 is the latest in a more than 20 year sequence of failures by US and Soviet nuclear satellites. Two previous RORSATs have re-entered the atmosphere. In 1978, Cosmos 954 scattered radioactive debris over 100,000 km<sup>2</sup> in northwestern Canada, and in 1983 a similar incident was avoided when the Soviets disengaged the reactor of the re-entering Cosmos 1402, allowing it to burn up on entry and disperse its radioactivity throughout the atmosphere.

The United States has had its share of nuclear satellite failures as well. Most of the 23 US nuclear-powered space missions have used radioisotope thermoelectric generators (RTGs), in which highly radioactive plutonium-238 generates thermal energy. In 1964, the aborted launch of a navigational satellite released 17,000 curies of plutonium-238 into the atmosphere. Three RTGs from aborted missions have been lost in the Pacific Ocean. Two were recovered, undamaged, but one, from the lunar probe aboard Apollo 13, has sunk irretrievably.

Whether the radioactive core of Cosmos 1900 will reach the Earth remains uncertain. The Soviets have suggested they may be able to disengage the core, but Western observers are sceptical, as radio contact with the craft has been lost. Current estimates suggest the satellite will come down in late September, but it could be brought down sooner by increased solar activity, which causes expansion of the Earth's atmosphere, or by loss of altitude control, which would result in uncontrolled tumbling. The satellite's orbital inclination of 65° takes it over virtually all inhabited parts of the globe, and it will not be possible until a day or so before its re-entry to determine the path of its fall. **Marcia Barinaga**

# Whither British science?

SIR—I wish to support what M.H. Dodson says on science teaching (*Nature* 333, 9; 1988) and to ask, now that the first examinations for the new General Certificate of Secondary Education (GCSE) have been held, whether people not teaching at this level are aware of what has been done in the name of improving British science education.

My personal commitment is to balanced science: all students should be exposed to the different disciplines of physics, chemistry and biology. Job advertisements for teachers show that the state schools have almost all adopted double award schemes, giving students two credits for 'science' rather than single credits for physics, chemistry or biology.

These double-award syllabuses have been supported by many distinguished bodies from the Royal Society down, so this is in no way surprising. Indeed, they are absolutely appropriate for most students and are a major advance for those who would previously have done little science after the age of 13, possibly even none. Yet I do not believe that this is a sufficient diet for able students whom we hope to encourage to study sciences later, at school and university.

To allow all science to be taught within 20 per cent of curriculum time, to accommodate the assessed practical components and to make the syllabuses and examinations accessible to all students, content has been diluted so that required knowledge is superficial — even in single subject syllabuses. Biology has lost its bite and chemistry has become a trivial pursuit. Physics is no longer mathematical.

Double-award schemes simplify still further, especially when they encompass geology, meteorology and astronomy. Time allocations and common papers for all abilities discourage the pursuit of any topic in depth. It is not the absence of particular facts that worries me, but the lack of concepts that will capture the imagination of able pupils and allow them to see that science is an intellectual challenge worthy of further study.

Many of the questions set in this year's papers are an insult to students of ability and can serve only to lower the status of science in their eyes. We old hands will continue to try to develop our pupils' appreciation and understanding beyond syllabus demands but it will not be long before most science teaching is aimed only at the levels required by syllabus and examination — especially by subject specialists teaching their two non-specialist sciences. Pupils of ability will be able to gain greater intellectual satisfaction from arts subjects such as English, history and foreign languages. There are no double awards here and teachers can still select *Macbeth* as a set book rather than *Adrian*

*Mole*. So my fear is that although the new schemes may encourage some pupils to take up science who would not have done so before (and who will almost certainly drop out when they find the going is tougher at Advanced level) we will at the same time cause many able pupils to choose arts subjects rather than sciences in the sixth form.

Finally, a reminder that what ultimately attracts able students to study a subject further is good teachers. The recent Smithers report on the shortage of physics teachers found no trained physics graduates teaching in any one of 28 randomly selected state schools for 11–16 year-olds. Teaching science in these schools rather than three separate sciences may be a pragmatic solution to the present problem but can serve only as a disincentive to graduates of quality who might otherwise have thought of entering the profession. A sight of some of this year's papers should be sufficient to frighten away any who may still have been hesitating. One physics question gave a velocity time graph for a parachutist. It showed a decreasing acceleration followed by a sharp discontinuity to terminal velocity without the parachute. Another sharp discontinuity as the parachute opened was followed by constant retardation to the lower terminal velocity! Simplification is one thing but asking questions about data that are patently nonsense to many candidates is inexcusable.

So my plea to those schools still free to choose is to hold the line for their best students, to stick with separate subject sciences, to teach beyond these and to press for examinations in which able candidates do not have to sit the trivial papers designed for lower ability candidates.

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## Polar observatories

SIR—Dreadco's daring projects as described in your journal's weekly column *Daedalus* have stimulated our imagination. An endeavour that would be of the greatest importance for the astronomical community has occurred to us. In spite of Dreadco's reputation, we do not hesitate to mention it in this letter (with the request to forward it to their research department); the profits made if the idea were realized would still be justifiable in view of the benefits for mankind.

It turns out that there are several compelling reasons for considering Antarctic mountains as sites for new astronomical observatories. With every significant astronomical organizations in the world planning 8-m class telescopes, the need for new high-quality and original

sites will soon become acute. The Antarctic offers itself through the availability of high mountains. Conventional astronomy in the visible would benefit because a location near the geographic pole makes possible continuous observations for many days or even months (of exceptional value for measurements of stellar oscillations and variable regions). The very low moisture content of the Antarctic atmosphere, as well as the natural near-cryogenic environment, would be optimal for infrared observations. The most significant advantage, however, lies in the revolutionary possibilities for ultraviolet observations directly from the Earth's surface. The ozone hole currently developing in the Antarctic, tragic though its consequences may be elsewhere, would greatly reduce atmospheric absorption in the near-ultraviolet. Dreadco's scientists may easily convince themselves that a reduction of the ozone content to 10 per cent (which may be realistic in the near future) might allow astronomers to extend observations by as much as 30 nm or more into the ultraviolet. Searches for very distant quasars would benefit especially at they are most easily recognized by features in this wavelength region. An extension of the size of the known Universe by as much as 50 per cent certainly seems possible. Finally, sociologists may be interested in studying the interactions taking place in a group of respectable quasar specialists working together for the duration of an Antarctic winter.

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## Wrong number

SIR—In the article "Scientists involved in Armenian environmental demonstrations" (*Nature* 332, 6; 1988), Vera Rich quotes Moscow radio world service's estimation of the ethnic composition of the Nagorno-Karabach region of the Soviet Union as 18 per cent Armenian. The truth is that radio reports announced 80 per cent Armenian, so the official commentators did not give "widely varying estimates".

The ethnic structure of the Nagorno-Karabach region, based on the most recent population census (1979) is: Azerbaijanis, 37,000 (22.8 per cent); Armenians, 123,000 (75.9 per cent); Russians, 1,300 (0.8 per cent); others, 700; making a total of 162,000 people.

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# Solar systems beyond our own

*The belief that our Solar System is only one of many seems to be confirmed in new measurements of the oscillatory motions of distant stars.*

## Baltimore

THE search for planets around stars outside our own Solar System has a respectable but erratic history. It once seemed that Barnard's star, a near neighbour, was orbited by a companion body several times bigger than Jupiter, a conclusion derived from astrometric measurements showing that the star wobbled slightly as it moved across the sky. But later observations failed to confirm the wobble, and the early measurements are now put down to inexact calibration, reflecting the Earth's motion around the Sun more than the orbit of a planet around Barnard's star.

Such cases of mistaken identification are far from uncommon in astronomy, but when they generate a great deal of excited publicity the subsequent disillusionment is chastening. This history clearly made Bruce Campbell, of the University of Victoria, British Columbia, and David Latham, of the Center for Astrophysics in Boston, circumspect in presenting to the General Assembly of the International Astronomical Union (Baltimore, 2–11 August) their respective claims to have found new planets.

As Colin A. Norman and Harold A. Weaver suggest on pages 474–475 of this issue, there are several reasons to suppose that our Solar System is not unique. Many young 'T-Tauri' stars have conditions conducive to the formation of planets, and presumably others have evolved to fully fledged orbiting systems.

There is no direct way to see planets beyond our own system. But searching for periodic oscillations in stellar positions, caused as the star and its companion revolve around their common centre of gravity, is almost a direct detection. If the period and amplitude of the motion can be measured and the mass of the parent star is deduced from its spectral type, the observer can calculate the product of the companion's mass and the sine of the angle at which the plane of the orbit is inclined to the line of sight.

A more far-ranging method is to monitor the spectra of stars, looking for periodic redshifts and blueshifts in certain sharp absorption lines, which would betray the presence of a regular orbital motion. But spectroscopically determined velocities have a typical error of 500–1,000 m s<sup>-1</sup>, whereas the motion given to the Sun by Jupiter has an amplitude of only 12 m s<sup>-1</sup>.

Latham and his colleagues have been

carefully observing several stars for some years, with the aim of beating down the errors by compiling large numbers of velocity measurements. The stars in their survey were chosen from lists of stars known to have small velocities, and their object was to determine the velocities with greater accuracy to establish the stars as reference objects. But one star in the survey disqualified itself by revealing a periodic motion.

The culprit is a Sun-like star called HD114762, in the constellation Coma Berenices. The measured amplitude of its motion is 726 m s<sup>-1</sup> which, as the typical error is assessed by Latham *et al.* to be 470 m s<sup>-1</sup>, may seem to make this a flimsy claim. But over 100 velocity measurements have now been made over a period of about seven years, and when the data are displayed against the derived periodicity of 84 days, a sinusoidal variation in velocity is unmistakably apparent. Furthermore, analysis of a similar series of observations of the same star by Michel Mayor at the Observatory of Geneva independently led to the same orbital velocity and period.

The short period puts the companion body in an orbit very similar to that of Mercury around the Sun, and the large velocity means that its mass (multiplied by the sine of the inclination angle) must be about ten times Jupiter's.

Although it seems clear that HD114762 has a companion, what is worrisome about the claimed discovery is whether the companion might not be a 'brown dwarf', more like a small star than a big planet. Anything bigger than about one-tenth of a solar mass, or 100 Jupiters, is a *bona fide* star; anything smaller than about 10 Jupiters is a true planet. The middle range is occupied by brown dwarfs, bodies which are too small to undergo nuclear burning, but which can reach a temperature of 1,000 K through internal heat generated by gravitational compression. Latham believes he has found a planet, but if the orbit is inclined at more than a small angle then the mass of companion must be correspondingly higher to produce the observed velocity, and the object may be a brown dwarf.

Recognizing the need for better velocity determinations, Campbell, with Gordon Walker and Stephenson Yang, has found a way to improve the precision of stellar spectroscopy by as much as one hundred times, although at the cost of restricting

their observations to bright, therefore nearby, stars.

Instead of recording spectra on a photographic plate, which is then removed from the telescope for measurement, Campbell passes the light from the star through a cell containing hydrogen fluoride, which superimposes its own spectral lines on the star's emission. This *in situ* calibration removes many errors, from thermal expansion and flexing, which arose in the old method as the spectra were taken and measured at different places and times.

By this simple expedient Campbell is able to measure velocities to an accuracy of about 13 m s<sup>-1</sup>, enough to reveal Jupiter's presence if he observed the Sun from afar. In a survey of 19 stars, this method has found measurable velocities, from tens to around 200 m s<sup>-1</sup>, in nine cases, and in one there are almost certain signs of a periodic motion.

In eight cases, Campbell finds stellar velocities that can be fitted, over the decade or so of observation, by straight lines or parabolas. If taken at face value as incomplete segments of long-period orbits, the planets responsible must have masses of 1 to 10 Jupiters. But no one will be convinced that eight new planets have really been found until the orbits have been followed for several periods.

Campbell's ninth set of measured velocities, of the star HR4112 or 36 Ursae Majoris A, constitute his best case. The star has a velocity which has changed in a roughly linear manner over ten years, but with a noticeable wobble superimposed. When the linear drift is subtracted, the orbit left has a deduced period of about 3 years and a velocity amplitude of about 20 m s<sup>-1</sup>, implying a planet about half as massive again as Jupiter. Scepticism over this 'discovery' arises because Campbell has many fewer data points than Latham, and has followed the orbit for less than three periods, compared with Latham's 30. But if the periodicity is confirmed, the companion to HR4112 is, by its low mass, undoubtedly a planet.

Irritatingly, Campbell's method will not work on Latham's star because it is too dim, and Latham's method is not accurate enough to see the wobble that Campbell detects. But both discoveries are promising, and Campbell's survey of 19 stars with nine detected motions raises the tantalizing possibility that about half of all stars are accompanied by planets of jovian proportions.

David Lindley

## Oceanography

# Deep-sea carbon, a burning issue

J.R. Toggweiler

ONE of the most heated debates in oceanography concerns the origin and characterization of the organic matter fuelling respiration in the ocean's interior. With information obtained from sediment traps in the past decade, most oceanographers have assumed that organic matter in sinking biogenic particles fuels deep-sea respiration. Now Sugimura and Suzuki<sup>1</sup> have identified a large class of previously unknown organic compounds dissolved in sea water.

These compounds seem to be produced by biota at the ocean surface and are broken down and metabolized in the interior, much like the particulate organic matter caught in sediment traps. But unlike sinking particles, they must be advected, or mixed, into the interior. Because they are dissolved, the compounds can be transported laterally over long distances from their sources by ocean currents before being broken down. Large gradients of dissolved organic carbon (DOC) beneath the ocean surface seem to mirror the downward increase in the amount of oxygen consumed from the water, suggesting that deep-sea respiration is fuelled mostly by dissolved organic compounds.

## High concentrations

Using a new technique for the oxidation of organic compounds in sea water, first described in an earlier study of dissolved organic nitrogen<sup>2</sup>, Sugimura and Suzuki<sup>1</sup> report that surface-water dissolved-organic-carbon concentrations in the western North Pacific are 250–300  $\mu\text{mol kg}^{-1}$  in contrast to the previously accepted range<sup>3</sup> of about 60–100  $\mu\text{mol kg}^{-1}$ . Deep-water concentrations of 50–100  $\mu\text{mol kg}^{-1}$  are also well in excess of previously accepted values (30–50  $\mu\text{mol kg}^{-1}$ ). By fractionating the DOC into size classes, Sugimura and Suzuki show that more than 90 per cent of the additional DOC uncovered by their technique is composed of compounds with relative molecular masses over 20,000. Previous work<sup>4</sup> established that most of the DOC in sea water had relative masses of less than 5,000. The newly discovered class of large compounds makes up most of the vertical gradient in DOC between surface and deep water.

The technique developed by Suzuki *et al.*<sup>2</sup> involves the injection of very small seawater samples, 100–200  $\mu\text{l}$ , into a high-temperature catalytic combustion furnace. The most reproducible DOC measurements made previously were based on oxidation of several hundred millilitres of sea water, using high-intensity

ultraviolet light or wet oxidation with persulphuric acid<sup>5</sup>. Bulk DOC concentrations approaching the range reported by Sugimura and Suzuki have been measured using a dry combustion technique, in which sea water was evaporated to dryness and the organic matter in the remaining salts burned. The reproducibility of this technique is poor<sup>6</sup>, however, and the results were largely disregarded.

Previous work<sup>9</sup> has shown that the radiocarbon age of deep-water DOC extracted by the wet-oxidation or ultraviolet techniques is about 6,000 yr. This indicates that much of the DOC is extremely resistant to microbial oxidation, and is cycled through the ocean many times. No one has yet attempted to measure the radiocarbon in the additional DOC extracted by Sugimura and Suzuki<sup>1</sup>, but it is clear from the large DOC gradients in the thermocline of the ocean that the turnover time of this pool of material is no greater than the circulation time for water in the upper kilometre of the ocean — a few hundred years. The ultraviolet and wet-oxidation techniques seem to extract the most refractory DOC compounds in sea water. The additional DOC extracted by Sugimura and Suzuki appears amenable to microbial oxidation, yet, ironically, it is resistant to attack by ultraviolet light and strong chemical oxidants.

Dissolved organic matter is released by many organisms in the sea<sup>7</sup>, and microbiologists maintain that DOC provides the main substrate for bacterial growth in the ocean<sup>8</sup>. The primary dissolved organic exudates of phytoplankton and the main food sources for the bacteria are identifiable low-molecular-weight metabolites and polymeric carbohydrates<sup>7</sup>. These compounds are present in sea water in low concentrations, and most are produced and consumed within hours or days rather than years<sup>8</sup>. Most of the DOC oxidized from sea water using the bulk oxidation techniques is chemically unidentifiable and is often referred to as marine humus<sup>9</sup>. One of the most thorny questions about DOC concerns the transformation of the relatively simple compounds produced by marine organisms into the complex, long-lived compounds which form the bulk of the dissolved organic matter in the ocean<sup>7</sup>.

At a meeting\* held to attempt an intercalibration of different DOC extraction techniques, it was clear that Suzuki's oxidation column could extract large amounts of DOC from water samples previously irradiated with ultraviolet

light, but no one could fully reproduce his results. J. H. Martin and S. Fitzwater (Moss Landing Marine Laboratory) have since acquired some of Suzuki's catalyst and have analysed ocean samples off California. They report DOC concentrations in surface water above 200  $\mu\text{mol kg}^{-1}$  with minimum values in deep water of 70  $\mu\text{mol kg}^{-1}$  (J.H. Martin, personal communication). Although these values are of the same magnitude as those reported by Sugimura and Suzuki<sup>1</sup>, the correlation between DOC and dissolved oxygen is quite weak compared with the correlation observed by Sugimura and Suzuki in the western North Pacific.

## Variations

Martin and Fitzwater's results could indicate real oceanographic variability or could reflect problems with the technique. P. Brewer (Woods Hole Oceanographic Institution) points out that no one really knows at present what reactions are going on in the catalyst tube (personal communication). Until these reactions are understood more fully, reproducibility between laboratories may remain elusive.

The significance of Sugimura and Suzuki's work stems from the implications of an oceanic DOC pool which is larger and turns over faster than was previously believed. If the mean lifetime of the additional DOC is about 100 yr, the mean production rate of carbon would be about 1  $\text{mol m}^{-2} \text{yr}^{-1}$ , which is comparable to particulate carbon fluxes at the base of the photosynthetic zone in the open ocean<sup>10</sup>. The production and dispersal of Sugimura and Suzuki's DOC thus represents a potentially significant fraction of the organic material exported from the upper ocean by the biota. A rough extrapolation from their published data shows that the ocean's DOC pool is about twice the size of the atmospheric  $\text{CO}_2$  pool and about twice that of the plant carbon biomass on land<sup>11</sup>. No one can say what constrains the size of the DOC pool. If the pool were to grow or shrink, the uptake or release of carbon should be reflected in changes in atmospheric  $\text{CO}_2$  concentrations. □

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\* US Global Ocean Flux Study workshop, Woods Hole, 17–20 November 1987.



# In search of a template

In contrast to most eukaryotic cells, trypanosomes and other kinetoplastida have only a single mitochondrion, the kinetoplast. When *T. brucei* grows in the bloodstream of a mammalian host, the single mitochondrion is reduced to a peripheral canal with few cristae; cytochromes are absent, the Krebs cycle is nonfunctional, and energy derives from highly efficient glycolysis performed by the glycosomes, specialized cytoplasmic organelles which

are surrounded by a lipid bilayer<sup>7</sup>. After ingestion into the midgut of the blood-feeding tsetse fly, the single mitochondrion slowly develops extensive cristae and metabolism comes to rely on respiration. Curiously, the apparent editing of some but not all mitochondrial transcripts in *T. brucei* is developmentally regulated (see ref. 1 for a review); this further complicates an already bewildering story.

The kinetoplastid mitochondrion contains relatively few (20–50) homogeneous maxicircles, thought to be analogous to orthodox mitochondrial DNA genomes, and a much larger number (approximately 5,000–10,000) of more variable minicircles that seem to lack protein-coding capacity<sup>8</sup>. The maxicircles and minicircles are concatenated into a single, dense network called the kinetoplast DNA. Concatenation may be facilitated by bent DNA sequences in minicircle DNA<sup>8</sup>, and perhaps also by the ability of G-rich homopolymer regions to associate with each other<sup>9</sup>. Why the maxicircles and minicircles are concatenated is a mystery. One novel possibility is that the concatenated kinetoplast DNA may serve as a molecular prison for mitochondrial DNA: the thousands of minicircles would in effect be jailors that keep the maxicircles from escaping to establish supernumerary mitochondria.

Orthodox mitochondria possess a complete protein synthetic apparatus and the mitochondrial genome encodes most of the organellar RNAs, including ribosomal, transfer (t) and mRNAs. RNA can, however, traverse the mitochondrial membrane, and the nucleus encodes at least two essential mitochondrial RNA species, a small RNA which is involved in mitochondrial RNA processing<sup>10</sup> and the RNA component of the mitochondrial DNA primase (D.A. Clayton, personal communication).

Admittedly it might seem perverse to question whether kinetoplastid mitochondria are capable of protein synthesis, but this has never been directly demonstrated. Neither ribosomes nor tRNAs have been isolated biochemically and computer analysis has failed to identify tRNAs in maxicircle DNA, although more than 95 per cent of the *T. brucei* sequence has been completed. The lack of identifiable tRNAs can be rationalized, given the highly divergent nature of mitochondrial tRNAs in the nematode worm *Caenorhabditis elegans*<sup>11</sup>, but the absence of ribosomes and failure to demonstrate protein synthesis are more puzzling. Furthermore, the 9S and 12S RNAs encoded by kinetoplastid maxicircles are the two most divergent ribosomal RNA homologues known (with sizes of 611 and 1,150 nucleotides, respectively, compared with 1,542 and 2,904 for 16S and 23S RNA in *E. coli*)<sup>12</sup>.

Although the 9S and 12S RNAs have

regions of sequence and structure that are universally conserved among ribosomal RNAs, it is worth keeping in mind that this could reflect their descent from ribosomal RNAs rather than their contemporary function. Recall that components of the protein synthetic apparatus may have functions apparently unrelated to protein synthesis. For example, ribosomal protein S1 and the elongation factors Tu and Ts comprise three of the four subunits of the RNA replicase of coliphage Q $\beta$  (ref. 13); and autocatalytic excision of a *Neurospora* mitochondrial group I intron *in vivo* requires a functional tyrosyl-tRNA synthetase<sup>14</sup>. Finally, the respiratory chain subunits (deduced by conceptual translation of kinetoplastid maxicircles using an orthodox mitochondrial genetic code) are exceedingly divergent from the homologous mitochondrial proteins of other organisms.

### Is there an RNA genome?

Rather than abandoning the central dogma, we are intrigued by the possibility that an RNA template within the mitochondrion directs synthesis of the edited COIII mRNA. Such an RNA genome would have escaped detection by the experiments reported thus far<sup>1–3</sup>. RNA would have been destroyed when total cellular DNA was incubated at 60 °C in 0.3 M NaOH before slot blotting; the RNA isolation procedure (urea-SDS followed by phenol-chloroform extraction) might have failed to deproteinize ribonucleoprotein complexes, which would then be lost on buoyant density fractionation in CsCl; primer extension analysis using an edited oligonucleotide would be unlikely to distinguish the plus strand of such an RNA genome from its transcript; and the published papers do not cite attempts to detect an RNA minus strand by probing with the edited plus strand. The difficulty of detecting a potentially scarce minus-strand RNA template must be emphasized, as replication of RNA coliphages, for example, is known to be extremely asymmetric<sup>15</sup>. In this context, it is worth noting that the existence of RNA plasmids in corn mitochondria has been reported by one laboratory<sup>16</sup>, and that the Mauriceville and Varkud mitochondrial plasmids of the fungus *Neurospora* resemble retroviral replication intermediates<sup>17</sup>.

If COIII mRNA is transcribed from an RNA template, the problem remains of explaining the existence of the unedited maxicircle COIII 'gene'. We suggest that information from the COIII RNA genome flows back into the maxicircle DNA by a process resembling gene conversion, and that thymidines tend to be deleted from maxicircle DNA when not maintained by selection. Nothing is yet known about the forces that maintain the peculiar base composition and extra-

ordinary strand bias typical of maxicircle DNA<sup>8</sup>, and in the light of this sequence bias, deletion of unselected thymidines would not be surprising.

Flow of information from RNA into DNA would also explain a critical fact which Feagin *et al.* do not address. At more than 80 per cent of the positions in the mature COIII mRNA of *T. brucei* where untemplated uridines are thought to occur, the COIII sequences of *Leishmania* and *Crithidia* maxicircle DNA also have thymidines (see figure). Conservation of thymidines between species would not be expected if the COIII genes of *Leishmania* and *Crithidia* were subject to selection only at the amino-acid sequence level. Transfer of COIII RNA sequence information to maxicircle DNA would avoid the need to postulate two parallel mechanisms, one for maintaining thymidines in DNA and the other for introducing uridines at the corresponding positions in RNA. This would imply either that information transfer from RNA to DNA occurred recently in *Crithidia* and *Leishmania*, or that the maxicircle DNA in these organisms is the functional (and perhaps the only) template for COIII mRNA synthesis. In contrast, information transfer would not have occurred recently in *T. brucei*, or the RNA genome in this organism would be such an effective template that there is no selection for maintaining a useful DNA copy.

Genomic plasticity is a hallmark of kinetoplastid protozoans. The absence of internal introns in nuclear genes<sup>18,19</sup> and the presence of retroposons<sup>19,20</sup> argue that much of the nuclear genome has undergone gene conversion templated by mature RNA sequences<sup>20</sup>. If the same is true for the mitochondrion, the kinetoplastid protozoans may be the closest approach yet to a living ribo-organism. □

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## Evolutionary biology

## The heritability of migration

William J. Sutherland

EVEN during his dying days, Gilbert White was still pondering over the conflicting evidence that birds such as swallows either migrate or overwinter in ponds<sup>1</sup>. Like other early scientists, he found it inconceivable that birds had the innate capacity to navigate vast distances. Although the existence of migration is no longer disputed, the manner in which migratory behaviour is inherited is little understood. A new and extensive series of breeding experiments by P. Berthold<sup>2-5</sup> on captive warblers shows that migratory behaviour is strongly heritable, and is likely to respond to environmental changes in breeding and wintering grounds. By a remarkable coincidence, the main species investigated showed a rapid change in migratory behaviour during the study.

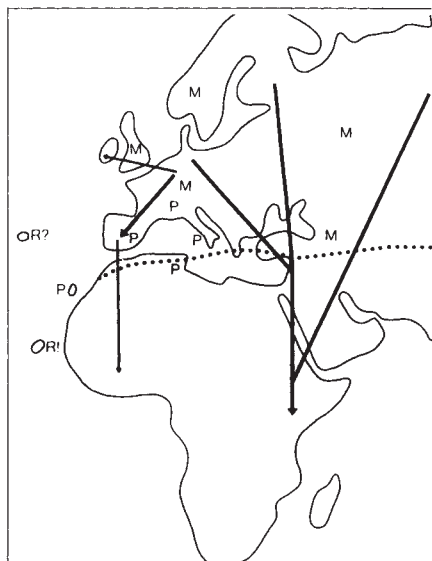
The relative importance of cultural and genetic inheritance seems to vary markedly between species. Among many species of wildfowl, the young migrate with the adults, and the young of pinnioned adults which cannot migrate do not migrate even if given the freedom to do so. This suggests that the migration pattern is learnt and helps explain the presence of traditional wintering sites for these species. At the other extreme is the cuckoo (*Cuculus canorus*) in which it is clear that the migratory pattern is innate as all the adults return south before the young have fledged from the nests of their foster parents<sup>6</sup>. Apart from such extreme examples, however, the relative importance of cultural inheritance and genetic inheritance is generally unknown.

Berthold's experiments elucidate the control mechanisms of the migration pattern in one group of birds. Members of the *Sylvia* genus of warblers show a remarkable diversity of migratory behaviour with different species undergoing long-distance migration, local migration and partial migration, whereas other species are sedentary. Berthold's experiments on captive birds suggest that the variation in migratory behaviour is linked to their endogenous rhythms<sup>4</sup>. Individuals kept under constant experimental conditions, with uniform day-length, temperature and food supply, show seasonal patterns of fat deposition and migratory behaviour. The different species of warbler show great variations in the timing and duration of migration which are closely mimicked by variations in their

endogenous rhythms. This same pattern applies within a species when comparing populations that differ in migratory behaviour.

Understanding the genetics of the system depends on three developments. First, it was necessary to develop ways of hand rearing and breeding considerable numbers of these insectivorous birds. Second, it was discovered that different populations of the blackcap (*Sylvia atricapilla*) display the whole range of migratory patterns present in the genus, so Berthold concentrated on this species. Third, he confirmed that the behaviour in cages is a reliable index of the actual migratory behaviour. Using electronic recording systems, the migratory activity of these nocturnal migrants can be measured as 'migratory restlessness', which can easily be distinguished from the diurnal activity. This measure assesses the extent and direction of migratory behaviour and so enabled the entire project to be carried out on captive birds. With these three developments, Berthold could cross-breed or selectively breed birds with a given migratory behaviour and then determine the migratory behaviour of the offspring. He was able to raise more than 1,500 warblers in captivity and tested them for migratory behaviour.

Crossing sedentary and migratory populations confirms that the differences in migratory behaviour have a genetic basis. The population of blackcaps on the Cape Verde Islands is fully resident and, as expected, did not show any migratory activity when transferred to cages in Germany. These birds were then cross-bred with migratory individuals from Germany and the  $F_1$  (first-generation) hybrids examined for migratory behaviour. Some (38%) of the hybrids exhibited



**Fig. 2** Distribution and migration of the blackcap in Eurasia and Africa. It breeds as far north as about 69° (in Scandinavia) and as far south as about 15° (on the Cape Verde Islands), and populations in this species are exclusively migratory (M), partially migratory (P) or (probably) resident (R?, R). Thick lines, main migration routes (to African and Mediterranean winter quarters); thin lines, by-routes (to west African and northern wintering areas); dotted line, southern border of the continental breeding grounds. (Courtesy of P. Berthold.)

migratory restlessness and the intensity of their migratory behaviour was 70% of the German migratory parents.

These hybrids also showed an orientation similar to their migratory parents. When tested in orientation cages, they concentrated their nocturnal activity along the same north-east–south-west axis used by their German parents on their regular migratory journeys. But their ability to head south in autumn and north in spring on that axis was not fully developed. The evidence suggests that the ability to orientate properly is genetically correlated with the migratory urge itself.

Partial migration, in which some but not all individuals of a population migrate, is a well-known phenomenon. Thus, some individual male Scandinavian chaffinches (*Fringilla coelebs*) migrate south while others remain on the breeding grounds. Two explanations of this phenomenon are first, that it is a stable polymorphism with genetic differences between migratory and non-migratory individuals<sup>7</sup>; and second, that it is related to individual differences in competitive ability with subordinate individuals having to move further<sup>7,8</sup>.

Berthold's new experiments on captive blackcaps (see Fig. 1) resolve this question by showing that partial migration is a genetic polymorphism<sup>2</sup>.



**Fig. 1** Blackcaps feeding at the nest. (Courtesy of P. Berthold.)

Birds taken from populations that undergo partial migration show variation in migratory behaviour in captivity when kept in uniformly constant experimental conditions. Different individuals, and even different siblings, can be categorized either as displaying distinct migratory behaviour or as being migratory inactive. That these individual differences are more than laboratory artefacts is shown by comparing birds from different populations in the same uniform conditions. All the individuals from the migratory populations show marked migratory behaviour whereas those from sedentary populations do not.

Selection experiments show that the extent of partial migration can be rapidly modified. In these experiments, Berthold used birds from a population in southern France known to be partially migrant<sup>2</sup>. Individuals were categorized as migrants or non-migrants by their migratory restlessness, and birds of the same category were bred together. The offspring of the F<sub>1</sub> generation differed markedly between the two groups with the migrant line producing 83% migrants in contrast to the 48% of the non-migrant line. A second generation of selection resulted in greater differences, with 92 and 30% migrants, respectively. The non-migrant line was subjected to a further four generations of selection and the percentage of migrants dropped further, reaching 10% by the F<sub>4</sub> generation. Thus, only three or four generations of strong selection could turn this population of partial migrants into one in which either almost all individuals are sedentary or almost all individuals are migratory.

The rapid change in response to selection suggests that migratory behaviour is strongly heritable<sup>2</sup>. Pairs of migrants produce some non-migrant offspring and pairs of non-migrants produce migrant offspring, implying either that several gene loci are involved or that the interactions between genes and environment are important. Calculating the realized heritability as the response to selection divided by the selection differential gives a series of values of heritability, of which all the ones with a large sample size have a value of about 0.6. This result confirms that the trait is strongly heritable.

These studies suggest that migration patterns can change rapidly, an idea supported by the change in migration route of the blackcap during the 20-year period of Berthold's study. Birds from central Europe traditionally fly south-west to winter in Iberia, but since the 1960s an increasing proportion has switched to a north-westerly migration to winter in England and Ireland (see Fig. 2). Those blackcaps which spend the winter in Britain and Ireland are largely dependent on bird-table food in suburban gardens, and the change in migration pattern is probably related to the increase in such food over

recent years<sup>9</sup>. Blackbirds (*Turdus merula*)<sup>10</sup> and starlings (*Sturnus vulgaris*)<sup>11</sup> have similarly changed their migratory behaviour in response to man-made environmental changes in Europe.

The droughts in the Sahel zone also affected the populations of birds wintering in that region. Thus the numbers of white-throats (*Sylvia communis*) returning to Britain in the spring of 1969 had declined by 70% and have not recovered since<sup>12</sup>. The numbers of sand martins returning to Britain have dropped by 90–95% over the past 20 years<sup>13</sup>, with the two main crashes in numbers coinciding with droughts in 1968–69 and 1983–84. The widespread environmental changes in the breeding grounds and wintering grounds of many species due to excessive land use, deserti-

fication and change in climate may, when combined with strong heritability, result in some dramatic changes in migratory pattern. □

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## Economic geology

# Silicates flushed with copper

Spencer R. Titley

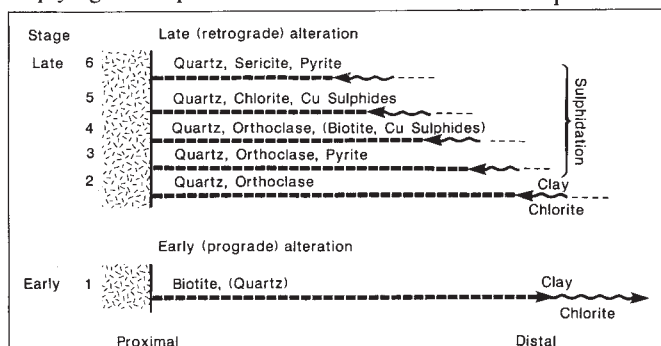
CONTRARY to conventional wisdom, biotite micas are the sink for leached copper in hydrothermal fluids, not the source, according to Ilton and Veblen on page 516 of this issue<sup>1</sup>. Their high-resolution electron-microscope and X-ray studies of samples from porphyry copper deposits reveal that copper is present as metallic, or 'native', inclusions between sheets of biotite. From their observations it may be possible to infer the route and original source of the copper, which should intrigue those who make a living from mining copper as well as mineralogists and geochemists.

Porphyry copper deposits are an important class of ores. Their most distinctive characteristic is their association with a specific clan of igneous rocks, implying that specific rocks and metals

share a common heritage. In the deposit type there is unequivocal evidence that enrichment with metal occurred after the formation of the rock which contains it. Copper is carried to the rocks by a hydrothermal fluid which is also the agent that causes the alteration of the pre-existing minerals<sup>2</sup>. But the metal source has been unknown. One possibility is that the metals and hydrothermal fluids are products of a residual solution in silicate melts: the orthomagmatic model. Alternatively, the metals could be leached and transported from various rocks, including igneous rocks, by water derived from magma, aquifers or sediments.

Orthomagmatic processes stem from the activity of melt-derived aqueous solutions originally rich in metals and sulphur, and require merely the release of such

fluids from specific, but poorly understood, silicate melts. The rocks in which the metals and sulphur are deposited are, in the extreme case, little more than passive receptors, and only the evolution of the igneous rocks affects the metal source and transport<sup>3,4</sup>. Processes dominated by leaching are revealed in the way that large volumes of rock surrounding intrusions are altered and mineralized. Modelling of the thermal history of large intrusion-



Mineralogical characteristics of laterally developed alteration stages recorded in deep porphyry copper systems formed in potassium silicate rocks. Alteration extends from an igneous intrusion (pattern) into wall rocks. The vertical axis represents time, the horizontal axis lateral changes (no scale). The inward formation of clays and chlorite is believed to result from the inward flow of water derived from sediments or aquifers during late cooling of the system. The mineral associations of hydrobiotite and native copper are permitted in the distal parts of early (hot) alteration (stages 1 and 2). Later stages (3–6) are characterized by formation of metal sulphides.



centred hydrothermal systems shows that the fluids must have moved laterally<sup>5</sup>.

Porphyry copper deposits from many regions reveal characteristics of mineralogy and geology indicating that they are formed by closely similar, if not identical, processes, and Ilton and Veblen's results are not unique to the deposits they have studied. It seems that copper is precipitated during a time of hydration of biotite to form "hydrobiotite". This would have been accompanied by a considerable flow of fluid through the system and the stabilization of the hydrous-alteration minerals. The introduction of copper during alteration of micas by hydration is consistent with larger-scale observations elsewhere<sup>6,7</sup> in which precipitation of copper sulphides accompanies the alteration of biotite, although this does not explain why the copper in Ilton and Veblen's samples is present in its reduced native form.

Ilton and Veblen did not detect significant volumes of copper in fresh biotite, so that biotites at the depths sampled are probably not a significant source of copper for leaching by hydrothermal solutions in primary ore-forming processes. But deeper, large volumes of biotite with low concentrations of metal could have been a source of leached copper. The absence of a sulphide phase in the specimens, which are believed to have come from beneath the zone of oxidation, which is the usual source of native copper, may indicate that copper and sulphur were introduced separately. As Ilton and Veblen note, late surficial weathering may be represented in the samples. Alternatively, the copper present could represent a precursor of still younger alteration characterized by sulphidation of metals.

As determined by Ilton and Veblen, the relationships between the textures of hydrated biotite and the character of native copper suggest synchronicity of a particular style of alteration and metal deposition not hitherto recognized. Continued investigation of hydrothermal alteration and metallization at the scale now observable with electron microscopy will provide better crystal-chemical detail interpretable in terms of complex hydrothermal metal-forming processes. □

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## Epidemics

# Evolution of plague virulence

Richard E. Lenski

WHAT was the ancestral form of the human immunodeficiency virus (HIV)?<sup>1</sup> How did HIV become so virulent? We do not yet know the answers to these questions, but on page 522 of this issue<sup>2</sup>, Rosqvist, Skurnik and Wolf-Watz present an interesting hypothesis for the genetic basis of the evolution of virulence in a former scourge, the bubonic plague. They hypothesize that less virulent strains of *Yersinia pestis*, the bacterium that causes bubonic plague, were harboured by non-human hosts, rats and fleas, during endemic phases. Single point mutations could have given rise to hypervirulent strains,

consistent with its much greater virulence relative to *Y. pseudotuberculosis* and with its inability to grow invasively in mammalian cell cultures.

Wolf-Watz and colleagues have shown previously that virulence plasmids pYV019 and pIB1, carried by *Y. pestis* and by *Y. pseudotuberculosis*, respectively, have a high degree of homology<sup>6</sup>. Both carry the *yopA* gene, although only *Y. pseudotuberculosis* expresses the corresponding Yop1 protein<sup>7</sup>. In the work reported in this issue, Rosqvist *et al.* have sequenced the *yopA* genes from *Y. pestis* and *Y. pseudotuberculosis* and find only

IMAGE  
UNAVAILABLE  
FOR COPYRIGHT  
REASONS

*St Gregory's procession against the plague. From the Soane Book of Hours, ca 1500.*

which spread to cause the plague epidemics.

Rosqvist *et al.* base their hypothesis on their elucidation of the genetic determination of virulence in *Y. pseudotuberculosis*, which is closely related to *Y. pestis*. The two are essentially indistinguishable from DNA hybridization data<sup>3</sup> and *Y. pseudotuberculosis* infections in rats provoke immunity to *Y. pestis*. Previous work has implicated two outer-membrane proteins in mediating the invasion of mammalian cell cultures by *Y. pseudotuberculosis*: invasin, which is encoded chromosomally<sup>4</sup>, and Yop1, which is encoded by a plasmid<sup>5</sup>. Rosqvist *et al.* demonstrate that mutations in one or the other of the genes encoding these proteins have little effect on virulence of *Y. pseudotuberculosis* in mice. But when mice were administered bacteria containing mutations in both genes, that is, *Inv*<sup>−</sup> and *Yop1*<sup>−</sup>, the LD<sub>50</sub> went down dramatically, indicating a heightened degree of virulence. *Y. pestis* apparently expresses neither invasin nor the Yop1 protein,

15 nucleotide differences among 1,230 base pairs. One of these differences, however, is a one-base deletion that throws off the reading-frame in *Y. pestis*.

The non-functional *yopA* gene in *Y. pestis* is presumably derived from a functional ancestral state. When Rosqvist *et al.*<sup>2</sup> introduce the functional gene from *Y. pseudotuberculosis* into *Y. pestis*, they observe a corresponding reduction in the virulence of *Y. pestis*. Thus, *Y. pestis* has apparently undergone a mutation in the past that caused loss of function of the *yopA* gene, with a concomitant increase in its virulence. This supports the hypothesis that single mutations played an important role in triggering plague epidemics<sup>2</sup>.

But mutations alone cannot drive epidemics. The necessary genetic variability in the pathogen must exist and so must the appropriate selective conditions for the spread of hypervirulent mutants. Hence, there remains the equally perplexing question concerning the selective

pressures that were responsible for the increase in the frequency of hypervirulent strains, once they appeared by mutation. Indeed, for many years, conventional wisdom favoured the view that evolution would select those pathogens which had the least harmful effects on their hosts<sup>8</sup>. On the other hand, pathogenicity, or virulence, is often associated with transmission<sup>9</sup>. Mathematical analyses by May and Anderson<sup>8,9</sup> and by Levin and Pimentel<sup>10,11</sup> have shown that the evolution of pathogens is highly dependent on this coupling between transmissibility and virulence.

To see this, consider a simple equation for the rate of change in the number of infected hosts in a population:  $dY/dt = \beta XY - \alpha Y$ , where  $X$  is the number of susceptible hosts,  $Y$  the number of infected hosts,  $\beta$  is the transmission rate of the pathogen and  $\alpha$  the rate of mortality including that induced by the pathogen. If the number of susceptibles is very high, the rate of increase of the pathogen — as reflected by the rate of increase of infected hosts — will generally be maximized when the transmission rate is high, even if this entails a high rate of host mortality. In contrast, reduced virulence could be favoured if perpetuation of the pathogen depends on prolonged survival of infected carriers, as may occur when the number of surviving susceptible hosts is very low. In a sense, a critical factor from the perspective of the pathogen is whether there is ample opportunity for infectious exploitation of other hosts or whether being 'nice' to a current host is the best way of achieving maximal fitness<sup>12</sup>.

The dramatic declines of the human

population in Europe during the great plague epidemics of past centuries were presumably accompanied by comparable declines in the population of susceptible rodents. Not only might these epidemics have been triggered by the appearance of hypervirulent strains of *Y. pestis*, as Rosqvist, Skurnik and Wolf-Watz hypothesize<sup>2</sup>, but the declining populations of susceptible hosts may in turn have favoured less virulent strains.

In a similar way, mortality among groups at high risk for AIDS, together with changing patterns of behaviour, may radically alter selection acting on the human immunodeficiency virus (HIV). How that might effect the evolution of the virus is not clear, owing to our present ignorance. As May and Anderson<sup>13</sup> have emphasized, we do not even know if heterosexual transmission of HIV is self-sustaining in developed countries. □

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## Planetary systems

# Body building in the solar nebula

Colin A. Norman and Harold A. Weaver

New observations and theories discussed at a recent meeting\* are rapidly elucidating the way that planetary systems evolve. Models are being proposed that show great promise in explaining quantitatively how all the planets in the Solar System formed during the short lifetime of the solar nebula. It now also seems clear that other stellar systems have the conditions conducive to planet formation. Circumstellar disks seem to be a ubiquitous property of T-Tauri stars (young, variable stars) and millimetre-wave observations of at least one of these reveal evidence that the material surrounding the star is in keplerian orbits. Excess thermal emission observed around many nearby evolved stars, such as  $\beta$ -Pictoris, also implies that our Solar System is not unique.

The speed with which the solar nebula

condensed to form the planets and other bodies in the Solar System has been hard to explain. A late stage of many models involves the building of planets from aggregates of smaller planetesimals, none of which survives. G.W. Wetherill (Carnegie Institution) illustrated how gravitational focusing causes the largest planetesimal at a given heliocentric distance to grow rapidly while other planetesimals about 10 km across in the same region show much more modest or no significant growth. In the model he proposed with G. Stewart (University of Colorado), these runaways are more efficient the further away the planetesimals are from the Sun. Thus Jupiter, and probably the other giant planets, could have formed earlier than the terrestrial planets.

Rapid formation of the giant planets is needed so that they can accumulate their dense gaseous envelopes before the solar

nebula is disrupted. This is emphasized by the observations of T-Tauri and 'naked' T-Tauri stars indicating that disk disruption occurs on a timescale of  $10^3$ – $10^7$  years. Runaway accretion is not sufficient to make Jupiter on such short timescales if the standard model for the nebular surface density as a function of heliocentric distance is used. But D. Stevenson (California Institute of Technology) suggested that water condensation occurring at 3 AU (astronomical unit; 1 AU is the mean separation of the Earth and Sun) from the Sun can drive density enhancements in this region and possibly where Jupiter formed. If the nebula near Jupiter really did have these large surface densities, Wetherill and Stewart's model could form Jupiter on a timescale of  $10^6$  years (J. Lisauer, State University of New York).

S.J. Weidenschilling (Planetary Science Institute) pointed out that we still do not understand how 1–10-km planetesimals can form from submicrometre interstellar grains to create the initial conditions necessary for Wetherill's model. An earlier theory (Goldreich, P. & Ward, W.R. *Astrophys. J.* **183**, 1051–1061; 1973), in which a thin dust layer a few hundred kilometres thick but many astronomical units in diameter is produced in the early stages of nebular formation, is improbable because a fluid-dynamical shear instability of the Kelvin–Helmholtz type will disrupt the dust layer. Less specific models involving turbulence-driven build up of fractal particles were discussed by Weidenschilling and B.D. Donn (Goddard Space Flight Center). It is important that there is no evidence of planetesimals in the meteoritic record (G.J. Wasserburg, California Institute of Technology).

Planets that form in a dense gaseous disk accreting onto a central star can have a profound influence on the disk evolution. Once an object the size of Jupiter is formed, it acts as a strong wave, or even shock, generator and the resulting wave and shock structures can act to transfer angular momentum rapidly outwards in the disk and thus strongly enhance the accretion flow (S. Miyama, Kyoto University; R.B. Larson, Yale University).

Important new results are emerging from observations of disks around protostars. There is evidence that about half the young stars observed by S. Strom and colleagues (University of Massachusetts) can be inferred to have disks. New data (F. Walter, University of Colorado) indicate that, at some early stage, most T-Tauri stars have their disks blown away. These results rely on inferences made from spectral studies. But HL Tau is the only properly imaged disk out of about 100 objects surveyed (A. Sargent, California Institute of Technology). It was generally agreed that millimetre and submillimetre wavelength imaging of the outer regions of protoplanetary disks could give crucial

\*The Formation and Evolution of Planetary Systems, Baltimore, 9–11 May 1988.



information on the surface-density-radius relation and chemical composition through the study of different molecules.

The nearby star  $\beta$ -Pictoris has an extended infrared and optical source presumably made from dust grains reradiating the light from the central star. We see only a symmetric line of radiation extending from either side of the star which could arise from bipolar flow (G. Herbig, University of Hawaii), although a circumstellar dust disk is the most "natural" explanation (M. Jura, University of California, Los Angeles). The circumstellar disk could comprise very dark grains similar to carbonaceous meteoritic or cometary material (R.J. Terrile, Jet Propulsion Lab.; B.A. Smith, University of Arizona) or high-albedo, icy grains (P. Artymowicz, Space Telescope Science Institute).

The spatial structure of the disk leads F. Paresce and C. Burrows (Space Telescope Science Institute) to infer the existence of lunar-sized bodies in the inner 100 AU. Fascinating observations of gas falling onto  $\beta$ -Pictoris were presented by A. Vidal-Madjar (Institute of Astrophysics, Paris). The gas could result from the vaporization of kilometre-sized bodies as they came within 2 AU of the star, although further modelling is needed.

A bit closer to home, isotope anomalies in meteorites and the atmospheres of terrestrial planets give clues about the formation of the Solar System (R. Pepin, University of Minnesota). The hydrodynamic escape of atmospheric constituents can account for the present distribution of isotopes although a specific mechanism for the escape is still lacking. The high ultraviolet luminosity in an early phase of solar evolution could do the trick as the photons are absorbed in planetary atmospheres, heating them and driving the hydrodynamic flow. But because T-Tauri stars have opaque disks and naked T-Tauri stars show no evidence for such ultraviolet excesses, it is not clear that the required photons are available.

The composition of comets can be used to identify the chemical state of the solar nebula (B. Fegley, MIT; J. Lunine, University of Arizona). Despite the exciting results from the Halley rendezvous, there are still obstacles to this approach. Chemical fractionation effects, such as the efficiency with which certain gases can be trapped in water ice, make it difficult to find a unique solution of the composition and a direct, *in situ* analysis of a comet with the Comet Rendezvous/Asteroid Flyby (CRAF) mission may be essential. Many proposals were discussed, including the programme for the Hubble Space Telescope, which might just detect planetary systems around other stars. □

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## Perforin

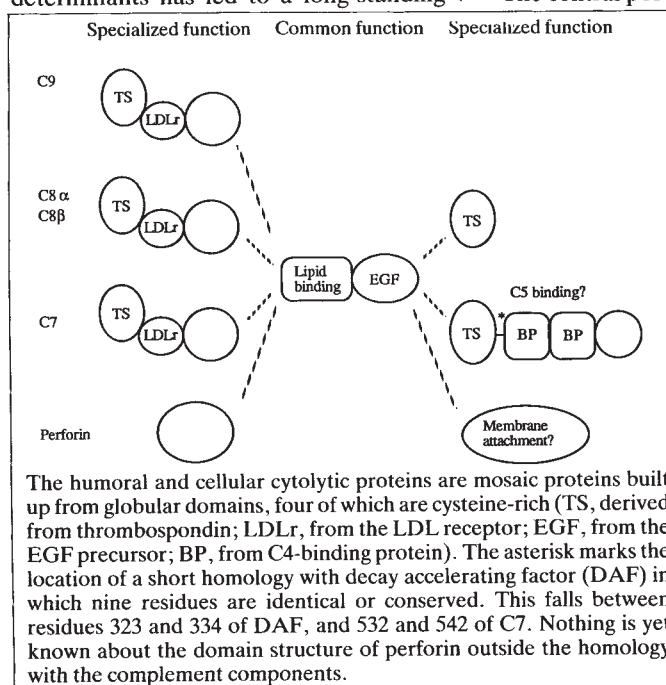
# A family of killer proteins

Keith Stanley and Paul Luzio

THE first report of the cloning and sequence of mouse perforin<sup>1</sup>, on page 525 of this issue, adds an important new member to the family of cytolytic proteins. Perforin is a granule protein of T-killer and natural killer cells which can form cytolytic pore-like lesions in a target cell. Although probably not the only mechanism of T-cell killing<sup>2</sup>, the similarity of pore morphology and shared antigenic determinants has led to a long-standing

(refs 12, 13) also share a high degree of homology to C9, because their principal function was thought to be making up the C5b-8 receptor for C9 insertion in the target cell surface. Only the sequence of C6 remains to be published but that too is expected to be like C9 (ref. 5). Comparing the shared homology among this family of proteins now reveals a fascinating picture of evolutionary cut-and-pasting.

The central portion of all the molecules



The humoral and cellular cytolytic proteins are mosaic proteins built up from globular domains, four of which are cysteine-rich (TS, derived from thrombospondin; LDLr, from the LDL receptor; EGF, from the EGF precursor; BP, from C4-binding protein). The asterisk marks the location of a short homology with decay accelerating factor (DAF) in which nine residues are identical or conserved. This falls between residues 323 and 334 of DAF, and 532 and 542 of C7. Nothing is yet known about the domain structure of perforin outside the homology with the complement components.

hypothesis<sup>3,4</sup> that perforin kills by a similar mechanism to the terminal components of the serum protein cascade known as complement<sup>5</sup>. The sequence now reported by Shinkai *et al.*<sup>1</sup> and recent work from Podack and colleagues<sup>6,7</sup> reveals that there is a genuine homology between perforin and the pore-forming C9 protein of complement<sup>8,9</sup>, although over a rather smaller region than was at first anticipated.

In contrast to perforin, which does not need additional proteins to insert into a target membrane, C9 is the last component of a membrane attack complex (MAC) made up of the five humoral complement components C5b, C6, C7, C8 and C9. The MAC is formed by a self-assembly process on the target membrane after complement activation has caused the production of C5-convertases that cleave C5 into C5a and C5b fragments. More surprising than the homology between perforin and C9 has been the revelation in the past year that the other proteins of the terminal complement cascade, C7, C8 $\alpha$  (refs 10, 11) and C8 $\beta$

is highly conserved (see figure). Its presence in C9 and perforin must account for the similar properties of membrane interaction and polymerization of the two molecules. Not surprisingly this portion contains the sequences that have been proposed on biochemical and theoretical grounds to form amphipathic structures that interact with the lipid bilayer. It also contains a cysteine-rich region with close homology to urokinase of the EGF family of cysteine-rich motifs. Presumably all the information for the

formation of stable cylindrical pores by these molecules is also contained in these two regions. C9 seems to be the simplest of the cytolytic proteins, with just three amino-terminal domains in addition to the common central portion. Two of these are cysteine-rich domains, one with homology to the LDL-receptor apolipoprotein-binding domain which has been implicated in cytolytic function. It might be this region that restricts C9 insertion into membranes containing C5b-8. The only difference in domain structure between C8 $\alpha$ , C8 $\beta$  and C9 is the presence of an additional domain at the carboxy terminus of C8 $\alpha$  and C8 $\beta$ , so this region probably accounts for the lack of polymerization of these two molecules. This domain appears to have been borrowed from thrombospondin and is found twice in C8 $\alpha$  and C8 $\beta$ , once at the amino terminus and once at the carboxy terminus.

As trout C9 contains the carboxy-terminal thrombospondin domain, which was not observed in the published sequence<sup>14</sup> because of a reading frame

error, this region must have been lost relatively late in evolution. If this domain does indeed restrict polymerization then MACs in trout should not contain multiple C9 molecules. In thrombospondin this domain is found on a part of the polypeptide chain involved in trimer formation, encouraging the interpretation that these regions in the complement proteins could be involved in forming the stoichiometric C5b-8 complex but they could prevent the polymerization that is characteristic of only C9 and perforin.

The largest terminal component of complement that has been cloned is C7. After the thrombospondin domain, this has two further cysteine-rich domains at the carboxy terminus, which are found in a large family of C3- and C4-binding proteins. This homology was not reported in the original paper<sup>10</sup> but both domains (residues 546-606 and 607-668) contain 15 out of 21 of the consensus amino acids for this family of sequences<sup>15</sup>. These sequences could neatly account for binding of the terminal complement components to C5b, which is an integral part of the terminal complex but resembles C3 and C4 in sequence. Acquisition of these C4 binding-protein-like domains explains how the early complement pathway, which opsonizes bacteria and produces inflammatory intermediates, has come together in evolution with the terminal pathway that provides a mechanism for membrane attack. Coupling the two pathways gives a specific stimulus to regulate the action of the humoral cytolytic proteins, analogous to the stimulus provided by effector-target cell interaction for perforin release.

The finding that perforin has a high degree of homology over a short part of the molecule, rather than an overall homology with the terminal complement components, should greatly help us to understand how cytotoxicity occurs. The mosaic character of these proteins, with many independently folding domains, means that the function of each region can now be ascertained by constructing chimaeric proteins. At the mechanistic level the questions can also now be more precisely formulated. How is the transition from a soluble protein to an integral membrane protein achieved? In perforin the presence of the same amphipathic helix thought to be used for membrane insertion in the terminal complement components strongly supports a two-step model. This involves an initial binding close to the membrane and then a refolding of the membrane-inserting domain to expose the hydrophobic patches<sup>14</sup>. It is still unexplained why this region allows the cytolytic proteins to bind to membranes with such avidity. Perhaps only site-directed mutagenesis can answer this question.

Perforin requires calcium for insertion into the membrane, which might facilitate

the initial binding of the protein in the proximity of the lipid bilayer. The carboxy-terminal domain of perforin<sup>1</sup> has some highly acidic regions which could account for calcium binding, although it has no recognizable calmodulin-like feature. For C9 the initial interaction occurs with the C5b-8 complex but, because we now know that this is composed of C9-like molecules, we are no nearer to explaining how the first membrane attachment occurs. The argument, which predated cloning, about exposure of hydrophobic regions directing the C5b-7 complex to the membrane no longer holds water, for none of the complement components is particularly hydrophobic and exposure of hydrophobic domains is anyway more likely to lead to aggregation rather than membrane insertion.

The proximity of the C5 convertase to the membrane surface is most likely to be equivalent to the calcium attachment of perforin but it is still possible that C6 or C7 has some additional mechanism for membrane attachment potentiated by formation of the C5b-7 complex. One possibility not yet investigated is the presence of covalently bound lipid. It is intriguing that there is a short region of the C7 sequence between the thrombospondin and C4 binding-protein-like repeats (asterisk in figure) that shows some similarity with the lipid-linked membrane binding domain of the complement regulatory protein, decay accelerating factor<sup>16</sup>.

The sequences of perforin and the terminal components of complement have provided many surprises. They are interesting proteins, not only because of their importance in immune defence but also because they provide a model for the molecular interaction of proteins with lipid bilayers. From the evolutionary point of view, it seems that the essential features of membrane attachment, membrane insertion and polymerization found in perforin have also been used to generate a series of specialized cytolytic molecules in the terminal complement proteins by the acquisition of protein domains conferring new properties. □

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## Daedalus

# Ocean currents

MUCH of the power needed to propel a ship goes into overcoming viscous drag. The water shrew solves this problem very neatly. Its oily fur repels water so perfectly that the creature as it swims is enveloped in a seamless silvery skin of entrained air and never even gets wet.

So Daedalus is devising a way of coating a ship's hull with a similar layer of drag-reducing gas. His ingenious strategy is to generate the gas directly on the hull surface by electrolysis, using the hull as an electrode. This will cover it with a uniform sheet of tiny bubbles, ideally suited as a captive overcoat. DREADCO's electrochemists are studying various electrode surfaces, from shiny and sticky finishes to rough, pitted or micro-bristly ones, seeking the best bubble-retaining characteristics. The final material should cling tenaciously to its bubbles, losing them only slowly by agglomeration and escape. So it will draw only a modest current, just enough to make up these gradual gas losses. In effect, it will be insulated from the water by its own gas coating. Only where the water breaks through this film will current flow: the resulting sudden burst of electrolysis will quickly push the water back again and break the circuit.

Any electrolytic cell needs two electrodes. So Daedalus's electrolytic vessel will be divided down the centre into two mutually insulated halves, port and starboard, with a potential of a few volts maintained between them by on-board turbine generators. The cathodic side will release hydrogen while the anodic side, in salt-water at least, will disengage oxygen and a little chlorine. (Both sides should powerfully discourage marine organisms.)

As a final brilliant touch, Daedalus will use his electrolytic drag-reduction system to propel his craft. The current will pass transversely beneath its keel, from port to starboard through the water. So Daedalus will mount big permanent magnets amidships, their fields penetrating the water vertically downwards. By standard motor action, they will force the current-carrying water astern, thus driving the ship ahead. Smaller control fields will deflect the water sideways to provide steering.

A ship coupled so elegantly to the water all around it would be wonderfully agile, manoeuvring in total silence with no turbulence at all. With negligible propulsive or viscous losses, it should also be amazingly economical on fuel. Mechanically simple and with no sonic signature, it should appeal to navies, too. Its small electrochemical losses, its weakly alkaline and chlorinated wake, and its complete inability to navigate by magnetic compass, are only minor drawbacks. David Jones



## Designing databases for molecular biology

SIR—I would like to endorse the approach recently proposed by Pongor<sup>1</sup> to the development of higher-order databases for molecular biology<sup>2</sup> and the view that artificial intelligence (AI) might be used in the study of macromolecular structure. This is, in fact, the motivation for some of our research in the ICRF Biomedical Computing Unit<sup>3</sup>.

One of the contributions that AI techniques make to our work<sup>4</sup> is to combine data retrieval and logical deduction within a common framework. It is my contention that extensive deductive databases, as such systems are called, will be a minimum requirement for the development of large scale knowledge-based systems in molecular biology. Knowledge-based systems are programs that can reason using representations of knowledge about the world and the ways in which knowledge is used to solve problems. Furthermore, the higher-order databases discussed by Pongor<sup>1</sup> and Pabo<sup>2</sup> will need to be knowledge-based if they are to achieve the required flexibility and extensibility.

In my experience, however, the development of extensive AI applications in molecular biology stretches present AI programming tools beyond their limits, in part because they are ineffective at complex deductive reasoning with large bodies of data. Research into knowledge-based management systems<sup>5,6</sup> is now being undertaken to alleviate this problem by combining the reasoning capability of AI programs with the efficient retrieval and management of shared data provided by database management systems. Another problem is that the AI techniques for representing some of the knowledge required for applications in molecular biology are relatively immature and require further research.

In addition to the problems of representational adequacy in AI techniques, there are significant issues that must be resolved in biology before a new generation of knowledge-based information systems for molecular biology can be developed. In order to represent a set of concepts in a computer, there must be an agreed syntax and semantics that are both consistent and complete. Agreements on the syntax and semantics of biological and biochemical concepts have previously been reached by nomenclature committees and workshops of the scientific unions (IUPAC and IUB, for example). But agreeing a nomenclature can often require considerable time, and clearly one that encompasses a significant part of modern biology is likely to require an enormous effort. Nevertheless, the development of a systematic ontology and epistemology of biology will be an important part of designing an integrated and comprehensive model for information

systems in molecular biology. These concerns make me disagree with Pongor's assertion that the concepts used to describe molecular biology can now readily be defined in unequivocal terms.

A next-generation molecular biology information resource should describe in a conceptual model the relationships among all the entities with data in the molecular sequence and structure data libraries as well as the higher-order relationships among sequences that were suggested by Pabo<sup>2</sup>. The model should be based on a systematic model of modern biology and should accommodate the views of data held by the different biological research communities — such as protein structure prediction, gene expression and molecular evolution. It must also accommodate uncertain or partial information and be easy to modify in the light of changes to our knowledge. Consideration should also be given to incorporating or referencing data collections of other kinds such as those listed in refs 7 and 8. It might also be feasible to integrate knowledge bases of experimental methods and computer software<sup>9</sup>, and it will be important that the scientific literature be available through abstract or bibliographic databases<sup>10</sup>.

This integrated information or knowledge source would be a powerful resource for biomedical research and its interdisciplinary nature demands that it be a collaborative venture; not just among biological scientists but also between the computer sciences and biology.

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## Lod score *Redivivus*

SIR—The proposal of Edwards<sup>1</sup> that 'log-likelihood' be used as a synonym for 'lod score' is analogous to suggesting that 'cat' be now known as 'monkey'. The advantages of such substitution are controversial, whereas the disadvantages are clear.

Wald<sup>2</sup> introduced the log-probability

ratio without naming it, and Barnard<sup>3</sup> coined the term lod for this statistic. Neither specified the logarithmic base in the definition, although Barnard assumed natural lods in his mathematical development. Several other synonyms have been proposed including log-likelihood ratio (consistent with the definition of likelihood but originally with a different meaning<sup>4</sup>). Edwards himself has previously suggested 'support' which is seldom used, perhaps because he equates it to log-likelihood when considering the probability under the null hypothesis as an arbitrary constant, and otherwise to the log-likelihood ratio<sup>5</sup>. In this he is unique, because the two statistics have different properties: by itself a log-likelihood ratio gives a test significance and a measure of information, whereas a log-likelihood does not. Of all the uncommon synonyms for lod (credibility, plausibility, information, weight of evidence and support), log-likelihood is the most idiosyncratic and the only one that is inadmissible.

Logic apart, use of lods is well established. The term is recognized in statistical dictionaries<sup>6</sup> and *Index Medicus*. Thousands of publications report lods, invariably to the base 10, a convention that was introduced by a member of Barnard's audience<sup>7</sup>. The advantage of common lods is that significance levels and conservative confidence limits ('support intervals') are easy to remember. For example, a lod of 3 corresponds to a significance level of 0.001, and a 1-lod interval to at least 90% confidence. In natural lods this translates to a lod of 6.9 and a 2.3-lod interval, respectively. A philosophical preference for natural lods hardly justifies a change from common lods or, what is worse, an ambiguous mixture of the two bases.

Edwards also objects to the word 'score', which entered the literature as tables for mating and ascertainment types ( $z_1$ ,  $z_2$  and so forth)<sup>8</sup>. Computer programs now make lod and lod score equivalent. By the phrase "score is now reserved for the first derivative of the log-likelihood" Edwards implies a consensus. On the contrary, eminent statisticians have termed this use "regrettable"<sup>6</sup>, and score continues to be applied in contexts as diverse as log-ranks and cricket.

Terminological quibbles never led to scientific advance. As Kendall remarked in discussion of the paper that sparked this controversy<sup>3</sup>, "all these people were really saying the same thing in rather different

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terms, or if they were saying different things it was only because they were talking about different things".

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## Enlarged family of putative helicases

SIR—I read with great interest T.C. Hodgman's correspondence<sup>1</sup> on the amino-acid motifs present in a superfamily of 21 proteins involved in nucleic-acid recombination/replication, comprising six separate families of related pro-

ribosome binding<sup>4</sup>.

The motifs noted by Hodgman are probably a feature of a very broad group of helicases rather than of recombination/replication-associated helicases. Helicases act in translation (eIF-4A), transcription (the terminator protein *rho*<sup>5</sup>) and may also act in RNA splicing reactions. It might be profitable to search for the motifs in all proteins known to be involved in these processes rather than just in proteins of suspected replicative function. Several known viral and prokaryotic helicases do not appear to contain all the motifs and further work will be required to establish if an alternate superfamily exists. *A priori*, one might predict at least two families based on the polarity of their move-

| I             | Ia      | II                                                                                                         | III                                                        | IV                                                | V                                  | VI |
|---------------|---------|------------------------------------------------------------------------------------------------------------|------------------------------------------------------------|---------------------------------------------------|------------------------------------|----|
| p68<br>eIF-4A | 7<br>58 | GVAQTSGKRTLSYL 20 LVLPAP-TRELAQQVQVA 56 YLVLEADRMEL<br>AQAQSGTGRTATFA 15 LVLPAP-TRELAQQTKRV 56 MEVLDEADEML | --DMGFEPQIRK 84 ETKRRCDL 10<br>--SRGFKDQIDY 81 NTRKRVDM 10 | ANGHGDKSQGE-RDWVLEEF 6<br>VSMANGDMQKE-RDWVHREER 6 | LITATDVASRG (4)<br>LITTLTLLALG (3) |    |

Conserved motifs of p68 and eIF-4A aligned to match the six families identified by Hodgman. As in Hodgman's figure (see *Nature* 333; 578), hyphens represent gaps introduced during the alignment and the number of amino-acid residues between motifs is shown to the left of each motif.

teins, to which I can now add a seventh, and the first higher eukaryotic, family.

Visual inspection of the deduced amino-acid sequences of eIF-4A, a translation initiation factor<sup>2</sup>, and p68, a nuclear protein that may be a helicase<sup>3</sup>, readily revealed the presence of all six of Hodgman's motifs in the two proteins, which share 32% amino-acid identity. As the figure shows, they also both possess the additional motif (here called Ia) between motifs I and II that Hodgman noted in a subset of the superfamily known to interact with DNA. In p68 and eIF-4A motifs II and III are fused and the gap between motif III and IV is atypically large, perhaps in compensation, because the gap between the II/III boundary and the start of domain IV is within the range of the other six families. The 92 residues of the motifs constitute 23% of the eIF-4A coding sequence and yet contain 41% of the total amino-acid identities with p68. Within the 92 residues, p68 and eIF-4A share 54% identity; outside the motifs they show only 24% identity.

The recruitment of this family of two new related proteins to the superfamily broadens its scope, as they are the only representatives of the superfamily drawn from the higher eukaryotes and because, while current work suggests that p68 is a DNA binding protein and may well be a cellular DNA helicase active in replication, eIF-4A does not have such a function. Instead, it is a polynucleotide stimulated ATPase, which exerts a helicase activity on double-stranded RNA and acts as an initiation factor for eukaryotic translation, probably by rendering the end of the mRNA single stranded to permit

ment along the polynucleotide backbone, but whether that is so awaits the determination of the polarity of more helicases.

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## Schistosomiasis vaccine

SIR—We read with great interest the letter entitled "Paramyosin and actin in schistosomal teguments" by Matsumoto *et al.*<sup>1</sup> In addition to corroborating published data on the presence of actin in tegumental spines<sup>2</sup>, this report confirms our own observation using monoclonal anti-schistosome antibodies which suggested that paramyosin may be localized in "sub-tegumental cells or . . . tegument itself"<sup>3</sup>.

We would like to correct a possible source of misinterpretation, however. While paramyosin is indeed located in the tegument, as Matsumoto and his colleagues describe, it is most definitely *not* exposed on the parasite surface in a manner accessible for antibody binding. We have made this observation repeatedly on intact worms<sup>4</sup> as well as worms damaged by brief exposure to praziquantel (P. J. Brindley, manuscript in preparation).

We have hypothesized that paramyosin functions as a vaccine antigen by being released from the parasite and eliciting a T-lymphocyte-dependent cell-mediated response rather than by serving as a surface-exposed target for protective antibodies<sup>5,6</sup>. In this context, the results presented by Matsumoto *et al.* are intriguing as they suggest a possible route (transport to the exterior via inclusion in membrane-bound elongate bodies) by which paramyosin may be liberated from the schistosome.

Regardless, it is clear from a recently completed vaccine trial that immunization with paramyosin can induce consistent protection against murine-schistosomiasis<sup>6</sup>.

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## Two chimps, too few

SIR—In their zeal to minimize the numbers of chimpanzees needed for AIDS vaccine tests (*Nature* 333, 513; 1988), Prince *et al.* disregard the biostatistical principles which form the basis of drug and vaccine testing. Acknowledging that it is "necessary to determine whether immunization is protective", they state that "This is most rapidly and economically done by immunizing two chimpanzees and then challenging them with a small dose of live virus to see if infection is prevented. If it is, clinical trials would ensue". I assume that they use the conventional definition of clinical trials, meaning tests in humans.

It is unfortunate for all who love animals that this sample size of two is not adequate for statistical inference. According to the elementary laws of probability, if a vaccine fails to confer protection in 10% of cases, we would need a study with 29 animals before we could be 95% certain of detecting at least a single such failure in our experiment. Indeed, if the vaccine fails 50% of the time, there is a 25% chance that both chimps in an experimental pair of animals would be successfully protected, as well as a 25% chance that both would fail to be protected. (I have assumed for simplicity an all-or-nothing response.) Thus if we make a decision based on two animals we may either move too confidently to clinical trials, or else erroneously judge a vaccine to be utterly worthless, and discard it. It seems to me terribly premature to move drug or vaccine testing from the preclinical to the clinical stage based on the slender evidence provided by small numbers of experimental animals, for to do so is to shift an unknown burden of risk from animals to human subjects.

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## The 4.9 per cent solution

*Laura Garwin*

**The Breakthrough: The Race for the Superconductor.** By Robert M. Hazen. *Summit: 1988. Pp.271. \$18.95. To be published in Britain on 22 September by Unwin Hyman.*

A SPEAKER at a recent superconductivity conference introduced a ball-and-stick diagram of a layered copper-oxide compound with the words, "Every schoolchild on the planet must know this structure". The structure was, of course, that of the '123' superconductor  $\text{YBa}_2\text{Cu}_3\text{O}_7$ , familiar to *New York Times* and *People's Daily* readers alike for its magical ability to conduct electricity with no resistance, simply by being immersed in liquid nitrogen.

It wasn't always so. Indeed, in January 1986 when Georg Bednorz and Alex Müller observed what looked like superconductivity at 30 K in a lanthanum-barium-copper oxide, the record transition temperature had stood at 23 K for over a decade. Superconductor research was a quiet, if fascinating, backwater of solid-state physics, and even true believers in 'high- $T_c$ ' superconductivity had begun to lose heart.

Unlike previous reports of superconductivity above 23 K, Bednorz and Müller's work was rapidly confirmed by two other laboratories — at the Universities of Houston and Tokyo. Paul Chu and Koichi Kitazawa made their announcements at the same symposium, on 4 December 1986. Suddenly the scepticism bred of a 13-year stall evaporated; perhaps liquid-nitrogen-temperature, or even room-temperature superconductivity was possible. Within days, laboratories around the world joined the race to higher temperatures, and there was talk of a Nobel prize for the winner.

But as Robert Hazen describes, Chu had already taken the crucial step towards higher temperatures before the 4 December meeting. He had subjected a sample of lanthanum–barium–copper oxide, with a stable superconducting onset at 32 K, to a pressure of ten thousand atmospheres, and had seen the onset temperature shoot up to 40 K. This suggested that higher transition temperatures could be achieved at room pressure, if the effects of high pressure could be mimicked by the substitution of atoms of smaller size. He enlisted his friend Maw-Kwen Wu, at the University of Alabama, in the search for suitable compositions, and on 29 January 1987 Wu's group observed the onset of superconductivity at 93 K in a sample of yttrium–barium–copper oxide.

Chu had finally broken the 'liquid-nitrogen barrier', but he had a problem: he did not know what the superconductor was. His sample contained at least two

different compounds, and no one in his team had the expertise needed to identify the superconducting phase and determine its composition and structure. Without knowing the composition, Chu could not prepare pure samples of the superconductor, or work towards improving its properties; without the structure, his theorist team-mate Art Freeman (at Northwestern University) could not perform the elec-

hadn't fully dried; the Polaroid X-ray camera that 'ate' more than half the exposures it took — made worse by the pressure of competition and necessity for speed. But eventually Hazen could "smell" the answer; the quality of the model fit improved steadily, from an 8% residual, to 6%, until one Sunday afternoon: "We solved the structure! 4.9% solution. Everything makes sense . . .".

Hazen is a good story-teller, and has a good story to tell; this thriller even has a spy, in the shape of Art Freeman, whom Hazen cold-shouldered for a week after hearing that he was collaborating with the 'enemy' at Argonne National Laboratory. (This rumour turned out to be false, and Freeman is exonerated in the book.) Hazen strives to convey the human side of

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# Houston Chronicle

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1. *Simulation of the growth of the human brain*

## Discovery may earn billions, Nobel for UH

**By CARLOS BYARS**  
Houston Chronicle Science Writer

Researchers at the University of Houston have grasped the Holy Grail of low-temperature physics, surpassing in a few months of intense work a goal long believed to be totally out of reach.

Their discovery of a superconductor that works at a temperature higher than liquid nitrogen could revolutionize the electrical industry, earn a Nobel Prize and be worth billions to the university.

Paul Chu, head of a team working to create new superconductors — materials that conduct electricity without resistance — said the new material operates at 98 degrees Kelvin. This is well above the critical 77-degree temperature of liquid nitrogen.

This temperature, though low by human standards

at minus 367 degrees Fahrenheit, is more than high enough to allow the use of cheap, readily available liquid nitrogen as a coolant. Previously, the temperatures required for superconductivity could be achieved only by using much scarcer liquid helium, virtually precluding any possibility of widespread commercial use of superconductors.

The discovery holds the promise of major advances in the transmission and use of electrical power, including better detectors of low-level heat and radio signals, more powerful magnets and motors and new tools for medicine.

Chu's discovery was announced here and in a scientific paper accepted for publication by *Physical Review Letters*, considered the most important journal of physics. This was the fourth major increase in the temperature of superconductivity in the last three months, after 13 years without a breakthrough in the

The discovery appears to have put Chu and the University of Houston far ahead of several well-funded, highly competitive teams. At stake is more than the intense competition for scientific honors and awards. The organization that wins clear title to the basic patents stands to gain license fees amounting to billions of dollars.

Sharing credit for the discovery was M.K. Wu of the University of Alabama, who was listed as co-author with Chu of a separate paper in the same publication announcing a superconductor at 93 degrees Kelvin. Wu is one of Chu's former students.

Further advances are expected. Roy Weinstein, UH dean of natural science and mathematics, said the group has seen signs that superconductivity occurs

**See SUPERCONDUCTOR on Page 5.**

*Houston hails Chu — but he didn't share in the Nobel prize.*

tronic band-structure calculations he needed to gain clues to the mechanism of the superconductivity. Chu had a month before his paper describing the synthesis of the 90-K superconductor would appear in *Physical Review Letters* — a month's head start before his rivals at IBM, Bell Labs and just about everywhere else would be able to make and analyse their own 90-K samples.

This is the race referred to in the book's subtitle, and this is where Hazen the narrator dons his running shoes. For Chu had decided to ask Dave Mao, a colleague of Hazen's at the Carnegie Institution's Geophysical Laboratory in Washington, DC, to help him identify the superconductor. On Friday 20 February, Mao enlisted Hazen to take charge of the X-ray crystallography, and from this point Hazen gives us a day-by-day account of the progress of his team, soon to number seven.

This narrative is the meat of the book, and it makes compelling reading. Step by step, the pieces of the puzzle fall into place, but against a background of all the blind alleys and wrong turns that never appear in published papers. Here are the everyday frustrations of research — the afternoon's work wasted because the glue holding the crystal in the diffractometer

scientific endeavour, often highlighting the inevitable conflict between work and family. Only occasionally does the personal element become intrusive; remarks such as, "The things I really wanted — the superconductor sample and Margee — were a thousand miles away" may be seen as concessions to a popular audience. On the other hand, that audience will surely benefit from Hazen's talent for making technical ideas understandable, often through the use of an apt metaphor. One memorable example is the description of the attempt to determine a unit cell from nine X-ray peaks as "something like having a sea urchin with all but nine spines missing; analysis of nine remaining spines might suggest positions of the missing ones".

The narrative culminates on 18 March, at the famous all-night session of the American Physical Society meeting in New York — the so-called ‘Woodstock of Physics’. Chu’s yttrium paper had appeared in the 2 March issue of *Physical Review Letters* and, somewhat to Hazen’s disappointment, several other groups were able to report the structure and composition of the 90-K (123) superconductor. Disappointment turned to consternation as the Bell Labs, Bellcore and IBM

*From The Breakthrough.*

Almaden groups all reported structures that were similar to the Geophysical Laboratory's, but which had orthorhombic rather than tetragonal symmetry. The difference in length of the relevant crystallographic axes was no more than one per cent or so, but Hazen knew that he should have been able to detect a difference of this magnitude. What had gone wrong?

A hurried discussion in the lobby with Robert Beyers of IBM Almaden, in which Beyers showed Hazen electron micrographs of highly twinned, orthorhombic 123, provided Hazen with an explanation just in time for his own talk. He proposed that the superconductor could adopt two different structures. Rapid quenching from high temperature yielded the higher-symmetry, tetragonal structure, whereas slower cooling allowed a structural rearrangement to take place, yielding the orthorhombic variant. The twinning seen in Beyers's micrographs was simply the crystal's way of adapting to the structural transition.

All this we now know to be true, and Hazen tells us in an epilogue that, after several weeks of worrying that they had somehow missed seeing the orthorhombic distortion in their crystals, the Geophysical Laboratory's structure was vindicated by other observations of tetragonal 123. What he doesn't tell us — a touch disingenuously, one feels — is that tetragonal 123 is not superconducting. He concludes, "To say that we 'won' seems, in retrospect, much too narrow a point of view"; in fact, if they won a race at all, it was unfortunately not the one they had entered.

While this error of omission will certainly not detract from the book's appeal for non-specialists, it may tarnish it somewhat for those in the field. Yet specialists will find much to interest them, including the fullest exposition yet of the notorious 'Yb for Y' episode — the substitution, whether accidental or intentional, of the chemical symbol for ytterbium for that of yttrium in the version of Chu's paper first submitted to *Physical Review Letters*.

As Hazen states in the preface, the book presents one participant's view of the race to solve the superconductor structure. Above all, it stands as an illuminating description of the "scientific symbiosis" between people with different knowledge and expertise that is becoming increasingly necessary as science grows more complex, and scientists more specialized. The field of high-temperature superconductivity is encouraging such symbiosis on a grand scale, as physicists find they can learn something from chemists, crystallographers and ceramists. It will be a long time (if ever) before the solid-state physicists have their backwater to themselves again. □

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## A wild social life

Adrian Lister

**Elephant Memories: Thirteen Years in the Life of an Elephant Family.** By Cynthia Moss. *Elm Tree Books*, 27 Wrights Lane, London/William Morrow, New York: 1988. Pp.336. £15.95, \$22.95.

CYNTHIA MOSS has devoted 15 years to a study of the elephants in Amboseli National Park, Kenya, during which time she has come to know each of the 600 or so animals as individuals. In *Elephant Memories* she describes in a most engaging way the fruits of this unique venture.

Elephants are highly social animals. They live in close-knit family groups led by an experienced female or 'matriarch', and associate to varying degrees with other families. Moss's first task was to

animal nor long-term cycles of climate and vegetation. Moss describes, for example, the way in which bond relationships and hierarchies evolve over the years and how they are affected by events such as the death of a matriarch; and the drastic shifts in patterns of migration, feeding and reproduction which were observed between years of drought and plenty. Previously contradictory conclusions about elephant behaviour and ecology are seen to be the result of studies carried out under varying conditions. The list of questions that can be answered by the data from long-term research lengthens almost exponentially with each year that passes.

Details of individual behaviour provide additional interest. 'Play behaviour', in which both adults and young participate, may involve throwing sticks, squirting water and running about in circles. Re-united relatives greet each other with much excitement and intertwining of



Tug of war — elephants at play in the water.

learn to identify all the animals in her study area, using particular features such as ear shape for recognition. Together with her colleagues, she could then begin to accumulate a vast body of data on patterns of association between individuals, daily and annual cycles of movement and feeding, reproductive behaviour and success, growth and development, and the effects on all of these factors of short- and long-term environmental change.

Each chapter begins with a brief narrative account of a period in the life of the study families, which illustrates a particular theme: drought, birth and babies, social relationships, population dynamics and so on. The reader is given a real feel for elephant life on a day-to-day basis in the wild. The chapter then continues with an extended discussion of its theme, incorporating the results of the scientific study. The result is an imaginative and highly readable text which will captivate non-specialists as well as providing a wealth of insight for professional ethologists, ecologists and conservationists.

It is the perspective of the years which gives the study its special importance. The very few comparable examples include Jane Goodall's work on chimpanzees and Fiona Guinness's on red deer. Projects such as these are simply irreplaceable by shorter-term studies, which encompass neither the full generation time of the

trunks. Animals which have died are covered with branches and dust by their relatives, who may keep vigil beside them for several days.

Finally, the book provides an informed and up-to-date account of the status of elephants in Africa as a whole, and the problems for their future survival. An estimated 80,000 of Africa's 800,000 elephants are now being killed annually for ivory, mostly by poachers. The establishment of game parks and reserves may ensure that the elephant survives, but Moss is critical of culling programmes which are often based on data collected at one particular time, and which may ultimately cause more ecological disruption than allowing natural regulatory mechanisms to operate. Her own studies of population dynamics in the undisturbed conditions of Amboseli will provide, for the first time, base-line data that should help in the conservation of all elephants.

Africa's elephants could have no more dedicated or persuasive champion than Cynthia Moss. Her whole book forms an impassioned plea for the preservation of conditions in which they can live their rich and complex lives to the full, as well as for the continued funding of research into these extraordinary animals. □

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# Back together again

C.J.H. Hartnady

**Geological Map of Sectors of Gondwana Reconstructed to their Disposition c. 150 Ma.** Compiled by Maarten de Wit, Margaret Jeffery, Hugh Bergh and Louis Nicolaysen. *American Association of Petroleum Geologists, PO Box 979, 1,444 South Boulder, Tulsa, Oklahoma 74101, USA: 1988. \$24.*

THE beginnings of this map-making effort go back to July 1979, when a 'Reunite Gondwana' workshop was organized by Nicolaysen and de Wit at the University of Witwatersrand. The first map compilation progressed rapidly, but for technical (and doubtless financial) reasons it has taken some years for the finished product to emerge. The wait has been well worthwhile.

The wall-map incorporates a long descriptive text in a left-hand column of rather small print. In addition to the main map, there are six peripheral inset diagrams illustrating the stages of Gondwana dispersion from 115 Myr BP to the present, and others showing the microplate structure of West Antarctica, an alternative tighter fit for Gondwana, a global distribution of Gondwana in Pangaeon times, and an apparent polar wander path for the southern continents relative to India. The legend is contained economically on the upper right-hand side, and includes a table of rotation parameters used in the computer reconstruction.

The palaeogeographic base is modified after the Norton-Sclater fit (1979), based in turn on the Smith-Hallam (1970) and, ultimately, the Alex du Toit model (1937). Some Earth scientists regard the Norton-Sclater reconstruction as being loose-fitting, and the small inset map shows an alternative tight-fitting Gondwana proposed recently by Lawver and Scotese. No attempt has been made to restore some orogenically shortened belts, such as the Himalayan and Andean chains.

I must admit to having some reservations about the straightforward representation of all post-Jurassic formations on a 150-Myr BP palaeogeographic reconstruction. Considering the authors' effort at interpreting or inferring the subglacial geology of Antarctica from very limited data, a similar attempt to strip off large areas of Cenozoic continental cover rocks in order to interpret and better represent the underlying geology would have been justified. Likewise, the map obviously lacks geological interpretation of some extensive continental-shelf and margin areas, where paucity of data is really not much of a problem.

On the other hand, I particularly appreciate the decision to include the post 150 Myr BP units. For example, given the current controversy over the role of flood-basalt volcanism versus meteorite impacts in causing biological mass extinctions such

as that at the Cretaceous-Tertiary boundary, I was interested to see the Deccan basalts (~65 Myr BP) represented along with the Parana basalts (early Cretaceous) and the Ethiopian basalts (Miocene-Pliocene) on the same equal-area map. The effect is to restore some perspective by deflating the 'uniqueness' of the Deccan eruptions.

Any user of this map will view it in the light of his own special interests, in my case, the Precambrian. There are a few places where the map has been overtaken by new data; the Gabon-Cameroon-Central African Republic region along the northern margin of the Congo craton is one example. Recent U-Pb and Sm-Nd geochronological investigations have revealed a belt of Pan-African granulite facies metamorphism — the Oubanguides belt — of late Proterozoic (640–840 Myr BP) age affecting a crustal protolith of early Proterozoic (1,700–2,200 Myr BP) age. Previous interpretations of this and geologically similar regions of Gondwana have almost automatically assumed that these granulites are of Archaean age, even when the available K-Ar and Rb-Sr geochronological data confirmed the nature and importance of Pan-African metamorphic imprints. Thus, the map confuses the Archaean and late Proterozoic granulite-charnockite bodies under the same colour code and chrono-symbol, and the tectonic boundary between the Oubanguides and Congo craton belts is blurred.

One wonders, however, if a large part of this problem does not lie with the authors' decision to abide by the convention whereby a background colour is used to indicate the protolith or crust-formation age of a metamorphic belt, and a superimposed pattern is used to indicate the metamorphic or tectono-thermal overprinting age. The alternative is a simple reversal of this convention.

In the case of the Pan-African crustal provinces of Gondwana, the alternative representation would undoubtedly emphasize their transcontinental coherence and continuity far more effectively. As things stand, the readability and intelligibility problems of 'overprint' patterning are compounded by variable quality of geological mapping and interpretations between different continents. Take the example of the Oubanguides boundary in Central Africa — it evidently must continue directly westwards into north-eastern Brazil as the boundary between

the Cairirian Province (Sergipano Fold Belt) and the Sao Francisco craton. Even though the South Atlantic strikes almost orthogonally across the structural grain of the Precambrian basement here — in itself, a geodynamically interesting fact — the age colours and patterning between the Archaean and late Proterozoic blocks on either side of the ocean are discordant. Perhaps the compilers were attempting to remain too true to their original source data, when the circumstances really required more interpretative licence.

This example, however, also illustrates the potential of the map for forcing a new look at old interpretations by lateral thinking on a transcontinental scale. One therefore looks forward to seeing the age-colour discrepancies between Nigeria and north-east Brazil disappear in future editions, as geologists on either side of the South Atlantic break through linguistic, politico-economic, geosemantic and other ephemeral barriers.

Problems of apparent geological discordance occur not only between continents, but also within them, in one case even across a minor body of water which also happens to be a political boundary. A wedge-shaped province of late Proterozoic age is shown extending southwards from the Arabian-Nubian shield into the northern part of the Mozambique belt. This is an important belt of Pan-African crustal generation and continental growth by island-arc accretionary processes, and its tapered termination on Lake Malawi's eastern shores raises many questions about the nature of proto-Gondwanaland plate tectonics. Is this where the East-West Gondwana collisional suture is really located? That the belt in question terminates opposite the extension of the Mwembeshi Shear Zone in Zambia towards the western shore of the lake, cannot be seen on the map because this particular transcurrent tectonic structure — a major geological boundary feature — is not represented. This example raises a far more general issue: whether or not the 'tectonostratigraphic terrane' approach to Precambrian crustal evolution, with its emphasis on the boundaries between 'suspect' blocks rather than the blocks themselves, should more consciously inform the compilation of geotectonic maps of this type. It again illustrates the special significance of late Proterozoic geotectonics in the origin of Gondwana.

Because of a decision, taken in 1984, to move away from conventional manual separation techniques to digital laser scanning technology for colour-plate production, users of the map will soon be able to acquire the 'Gondwana digital database'. Manipulation of the database by CAD software systems to produce derivative hard-copy images devoted to specific geological features or particular periods of geological time is one immediately

obvious spin-off. The integration of other detailed or local geological data-sets of different type (such as metallogenic) is another new possibility beckoning the Gondwana researcher.

The Reunite Gondwana project therefore seems set to lead from map to database and colour-graphics workstation display, from static picture to kinematic and selectively multi-faceted imagery. But although the wall-map may not have the

gee-whiz appeal of an electronic product, it will undoubtedly trigger intense study and reflection on some of the salient problems of how the Earth works. It deserves a prominent place in every Earth scientist's office, and in the foyers of all geological institutions. □

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## Reading palms

*Peter H. Raven*

**Genera Palmarum: A Classification of Palms Based on the Work of Harold E. Moore, Jr.** By Natalie W. Uhl and John Dransfield. *The L.H. Bailey Hortorium/International Palm Society*: 1987. Pp. 610. Distributed by Allen Press, Lawrence, Kansas. \$74.95. (Available in Britain from the Bentham-Moxon Trust, Royal Botanic Gardens, Kew, £45 plus postage.)

PALMS are second only to the grasses in worldwide economic importance and they dominate ecosystems throughout the warmer parts of the globe. A lifetime of work by Harold Moore, who died prematurely in 1980, provided the basis for the new classification presented in this volume. Although primarily taxonomic in scope, the book is so attractively presented and well organized that it assumes a general importance one would not necessarily associate with such a work.

In writing the book, the authors did not simply take over Moore's concepts and expand on the material available in his notes. Instead, they methodically reviewed all genera of palms themselves, reassessing Moore's views and often bringing their conclusions into better agreement with the evidence. In doing so, they drew upon their own considerable studies of the group, and were often able to improve greatly on the concepts available in 1980. Pointing out several areas where our knowledge remains inadequate for a definitive classification, the authors call for cladistic studies of the palms to improve both the classification and our understanding of evolutionary patterns in the family. It is also clear that the application of macromolecular studies to the further elucidation of relationships in the family could be undertaken with profit, using the classification presented here as a starting point.

Although some of the habit photographs could have been better, the way in which this volume presents the diversity of palms — plants that can be appreciated properly only in a living state — is truly impressive. In this connection, special mention must be made of Marion Ruff



*Palm habits — a reduced reproduction of Marion Ruff Sheehan's colour original.*

Sheehan's superb illustrations, which demonstrate the value of having an artist work consistently on a project of this sort.

In this classification, palms are divided into 200 genera. Thorough morphological and anatomical investigations of vegetative features, inflorescences, pollen, chromosome numbers, ecology and geography, based on travel throughout the tropics and subtropics, have led to the grouping of these genera into six sub-families and 16 tribes. The descriptions of the genera are clear and well presented, and there are ample diagnostic illustrations. A particularly useful innovation is the well-illustrated glossary, which imparts unusual clarity to the meaning of the descriptions.

In terms of species, palms constitute only about 1 per cent of the estimated 250,000 flowering plants. In terms of their role in the ecosystems of all warmer regions, however, they are much more important than that number would seem to signify. Because of that vital ecological role, it is especially gratifying that palms are the subject of such a landmark work. *Genera Palmarum* will stand as a key work of reference for decades to come, and it establishes a new standard for monographic treatments of the major plant groups. One hopes that it will be emulated often in years to come. □

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## With a little bit of magic

*M.J.A. Tanner*

**Immunochemical Methods in Cell and Molecular Biology.** By R. J. Mayer and J. H. Walker. *Academic*: 1987. Pp.325. £26, \$49.50.

Books describing practical techniques in the biochemical sciences are becoming increasingly popular, both because of the diverse range of methods in use nowadays and because pressure on space in journals rarely allows the inclusion of more than a bare outline of experimental protocols in papers.

Mayer and Walker's volume covers the range of methods which use antibodies that are now established as basic tools in biological research. The major portion of it consists of a review of the production and applications of both polyclonal and monoclonal antibodies, with sections on most of the immunochemical techniques in common use. The authors assume little previous knowledge of the subject and provide a very readable discussion of the topic.

There is still a little bit of magic in preparing good antibodies and getting them to work well. The empirical basis of many immunochemical methods often makes it difficult to define clearcut rules in applying them, and this is reflected in the book. The survey discusses the methods mainly by example, many of which are taken from the authors' own work. It also provides a useful summary of the variety of experimental conditions which have been employed with each of the techniques discussed. The review concludes with a chapter on synthetic peptides as antigens, which is written with great enthusiasm but unfortunately does not cover some of the newer developments in adjuvants for raising anti-peptide antibodies.

The final part of the book contains a collection of step-by-step experimental details for most of the methods described in the review. Surprisingly, no protocols for the preparation of monoclonal antibodies are provided, although the review does contain some relevant experimental guidance. Also omitted are details of the faster and more economical dry and semi-dry electroblotting techniques. But this part of the book provides a clear and detailed guide to a large number of methods, ranging from the raising of antibodies to immunohistochemistry on tissue sections. It will be a useful laboratory manual of immunochemical techniques, especially for those unfamiliar with the area. □

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# Modelling the future: a joint venture

David Rind, Arthur Rosenzweig and Cynthia Rosenzweig

*The consequences of a possible global warming are far from clear. Modellers of economic and climatic change need to put their heads together.*

DURING the autumn of 1987, two events occurred which highlighted our inability to forecast near-term anomalies. First, on 15–17 October an intense storm moved through western Europe, the strongest storm in the area for over a century. Hurricane-force winds disrupted electrical power and transport across southern England, and sea-level pressure in London dropped to 960 mb, the lowest on record. High winds and heavy flooding were experienced from Spain to Scandinavia<sup>1</sup>.

Then, some three days later, on 19 October the share prices of publicly traded American corporations as measured by the Dow Jones Industrial Average of thirty leading stocks dropped by roughly 500 points, or more than 20 per cent of total value. About 500 billion dollars were wiped off the slate. By all methods of accounting, it was the greatest asset 'melt-down' in financial history.

In addition to the proximity of these events in time, they have one other thing in common: in neither case was such an extreme occurrence forecast, and even during the events, meteorologists and economic analysts were incapable of keeping up with the rapid changes. The inability to foresee these extreme anomalies has properly raised doubts about how well experts in the two fields understand their subject, especially when dealing with situations outside the norm.

## Electronic computer

Both atmospheric and economic modelling became practicable with the advent of the electronic computer after the Second World War. John von Neumann, the computer pioneer, saw meteorology and economics as the two applied sciences which could most benefit from the power of computers because of the size and complexity of their models<sup>2</sup>. In recent decades, numerical forecasting in the two disciplines has proceeded with varying degrees of success, and without much interaction between them. Now, however, climate and economic modellers will have to combine to tackle a new forecasting problem: the effects of climate change on world economies.

Increasing concentrations of greenhouse gases in the atmosphere are expected to produce significant alterations in Earth's climate over the coming decades<sup>3,4</sup> (Fig. 1, see over). Although

researchers have focused on the problem of predicting what the climate changes will be, and to a lesser extent on estimates of future trace gas releases, so important to climate projections (Fig. 1), these efforts have not been interactive. Furthermore, analysis of the consequences of such changes for society lags far behind. In recognition of this deficiency, the World Meteorological Organization has called on governments to increase their support for analyses of the possible economic effects and appropriate policy options<sup>5</sup>. The Congress of the United States has asked the US Environmental Protection Agency to report on the potential effects of climate change and the possibilities for stabilizing climate. In response, the agency has initiated a programme which includes linking climate models with economic models. The results are due this autumn.

There are at least 20 global simulation models in use in each of the two disciplines, with less than half of the atmospheric models being used for long-term climate-change assessment. Are climate and economic models compatible? What are their respective strengths and weaknesses? Can we have any confidence in their joint prediction of the effects of climate change in the next century, when they cannot foresee record-breaking events that happen the very next day? We will address these questions through a comparison of the two modelling approaches, and suggest ways in which the disciplines can begin to work together.

The most striking difference between climate and economic models is in the relationship of the models to the basic principles of the disciplines. Climate forecasters use 'general circulation models' which solve the fundamental equations representing the well-established theories of conservation of physical quantities (mass, energy, momentum and moisture). At least ideally, they are operating directly from these first principles. For example, in order to forecast the coming climate change, a modeller increases the amount of CO<sub>2</sub> and other trace gases in the radiation scheme; this reduces the amount of energy leaving the atmosphere, which in turn alters the temperature and then other climate variables in the model such as winds, cloud cover and precipitation patterns. All that modellers require to generate the response of the system to an initial perturbation is the ability to calcu-

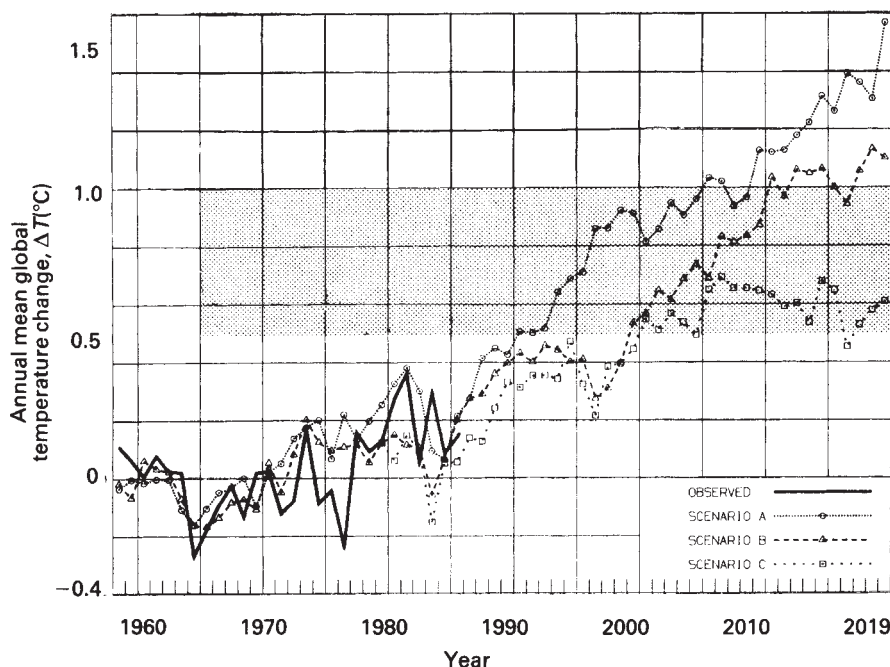
late the physical forcing terms in the fundamental equations, in this case absorption of radiation by gases in the equation for conservation of energy.

## Human choice

In contrast, economic principles do not yield fully closed sets of equations. Key relationships are 'behavioural', that is they represent processes subject to human choice. The functional forms and parameter values of behavioural equations cannot be derived from available theory. The generally accepted principles of economics, such as the laws of supply and demand, simply provide guidelines. For example, the law of demand implies that the demand for electricity is negatively related to its price and positively related to the prices of alternative energy sources. The quantitative functional form of these relationships cannot be derived directly from the principles.

In economic models, the completion of the specification of the equations — the functional form, the operational definitions of the variables and the values of the coefficients of the variables — is typically based on empirical data. The choice among the (perhaps infinite) universe of specifications that are consistent both with the economic principles and the data set available is made according to a conventional set of decision rules. Often the data are assumed to exhibit a specific pattern of random deviation from an otherwise deterministic relationship, so that statistical criteria can be used to derive and evaluate alternative specifications. This is the approach associated with 'econometric' modelling. Sometimes the data are used to obtain average values, or ranges of average values, of the coefficients of first- or second-order variables in linear equations. This approach is broadly descriptive of input-output, linear programming and systems dynamics models. In general, then, economic theory may suggest the direction (that is the algebraic sign) of a change in a behavioural variable (peak seasonal use of electricity, for example) in response to a change in conditions, but not the size of the response or the functional form of the equation, which have to be generated from data.

The importance of the role played by first principles in climate models should not be exaggerated, however. In atmospheric models, many of the physical forc-



**Fig. 1** Annual mean global surface air temperature change computed in the Goddard Institute for Space Sciences general circulation model for three different scenarios of trace gas growth<sup>9</sup> (A assumes current growth rates of trace gas emissions; B assumes decreasing growth rates; C assumes no trace gas increase after the year 2000). Observational data is from Hansen and Lebedeff<sup>10</sup>. The shaded range is an estimate of global temperature during the peak of the current and previous interglacial periods, about 6,000 and 120,000 years before present, respectively. The zero point for observations is the 1951–1980 mean<sup>10</sup>; the zero point for the model is the control run mean, with 1958 trace gas concentrations. The results show how sensitive predictions of future temperatures are to the future releases of trace gases and thus to the future economy.

ing terms (for example, turbulent fluxes) are imperfectly known, and this is even more true in representations of other components of the climate system, such as the ground hydrology and oceans. Therefore climate modellers also have to choose the functional relationships of various processes by reference to observations. In the physical sciences, controlled experiments, in which only one parameter is allowed to vary at a time, can often be conducted to derive the functional relationships; thus atmospheric modellers can carry out experiments in some cases (for example, determining absorption spectra of atmospheric gases). But this cannot be done generally, for it is impossible to hold climate variables fixed in the open physical system of the real world.

In economics, as in the other social sciences, controlled experiments are difficult, either because the variables cannot be controlled or because it is prohibitively expensive to do so. Thus, although basic principles and, to a lesser extent, controlled experiments are sources of specific formulations for climate models but not for economic models, both ultimately contain functional relationships derived from uncontrolled observations. Furthermore, the necessary reliance on observations raises the possibility that both models are 'tuned to' (that is, biased in favour of) the current situation.

Spatial scales in climate models are

determined by the 'grid' over which the model calculations are made, generally in the order of  $10^4$ – $10^6$  km<sup>2</sup>. 'Sub-grid' scale processes are incorporated in parameterized form, based on field measurements or mesoscale models, while larger scales are all explicitly represented in the continuous global model. Although there are different models for different scales, climate and climate change are global phenomena involving energy transport from one region to another, and must be addressed with global models.

Economic models, instead of using gridded spatial dimensions, are organized in terms of scales of economic activity. The equations of microeconomic models represent the activity of an individual agent (buyer or seller), a group of agents (for instance, electrical utilities), a market (supply and demand for electricity) or a sector (energy). Macroeconomic models represent activity at higher levels of aggregation, defined in terms of an appropriate geopolitical unit (county, state, country, groups of countries), a unit of collective decision-making. Economic data (and thus model validation) tend to be reported in geopolitical units, rather than in uniform geographical scales. In assessing the impact of climate change, the microeconomic approach is necessary for studying the direct effects at the point of contact between climate change and economic processes, but the macro-

economic approach is needed for evaluating overall costs and benefits within the geopolitical area of relevance to policy-makers.

Economists use very different models for different time-scales, reflecting their belief that the effects of some variables are more important in the short term but are dominated by the effects of other variables in the long term. Short-term macroeconomic models, which simulate quarterly changes over a period of about three years, involve financial variables such as money supply, short-term interest rates and consumer credit, as well as changes in inventories and profits. The model-builder's objective is to track cyclical fluctuations in production, income, employment and spending. These models are usually incapable of generating cycles beyond a single turning point, such as the end of a recession, and tend to diverge, producing exaggerated, unrealistic results if run over long periods.

Longer-term economic models employ a completely different set of variables: demographics, capital accumulation, the consumption of natural resources and technological change. They are used to simulate annual changes over periods from five years to several decades and provide the basis for strategic planning decisions.

In theory, atmospheric models can be used for both short-term weather forecasting (days to weeks), or longer-term climate projections. The same fundamental set of equations applies regardless of time-scale, because climate is simply the time-averaged weather. Additional physical processes become important on time-scales of years to decades, such as the transport of heat through the bottom of the oceanic mixed layer or the effect of changing vegetation. Other components of the system, such as ice sheets, influence climate on time-scales of centuries to millennia. Representations of these effects must be included in climate models when relevant, so climate modelling involves additional scientific disciplines compared to weather forecasting. But such processes still act on the primary atmospheric variables of temperature, pressure, wind and moisture, which are the same regardless of length of time of integration.

## Time and space

In practice, however, weather-forecasting models are never used for long-term climate studies, nor are climate models used for short-term predictions. Weather-forecasting models are run with much finer spatial scales to guarantee proper movement of weather systems. In order to minimize the time it takes to produce forecasts, these models do not include processes which act too slowly to influence weather. They thus often lack the requisite physics for long-term integrations,



and their numerical schemes do not necessarily conserve fundamental quantities such as mass and energy. In some cases, they too will diverge if run for a long time. On the other hand, the general circulation climate models are integrated for up to one hundred model years and are developed on coarse spatial scales. When run with finer resolution they eventually develop excessive winds, presumably due to the parameterization of processes not fully understood. Both climate and economic models are thus developed for and tuned to specified time and space horizons.

## Forecasting

Climate and economic modellers have somewhat different forecasting methods. A climate model, like most physically based models, provides a single-valued solution when posed a specific problem; that is, one climate forcing function (such as  $\text{CO}_2$  concentration) is changed and the model provides a specific 'forecast' of the other internal climate characteristics (temperature, precipitation and so on). The important variables which are not predicted and have to be specified, such as solar radiation, are thought to be limited in number.

Economic models tend to contain a greater number of important variables whose values are not determined, and therefore need to be pre-specified. In a macroeconomic model, these variables usually represent policy decisions, such as the level of government spending or the growth rate of the money supply. In a microeconomic model, they usually represent variables that would be determined at a higher level of aggregation, such as the world price of wheat in a regional agricultural model. So each model simulation requires assumptions to be made about the variables whose future values are exogenous (external) to the model, and economic models thus produce results which are often cast in terms of probability solution sets. Actually, both climate models and economic models produce ranges of results: given the uncertainties in projected emissions of trace gases (which depend upon economic factors<sup>6</sup>), there are many alternative climate-change scenarios, each producing a specific forecast for use in economic assessments, with each forecast then leading to a probability distribution of economic model output.

What is the current status of the different forecasting disciplines, and how much confidence can we place in their predictions? If the use of empirical relationships implies that climate and economic models are biased towards representing current conditions, then there is a fundamental question regarding their accuracy when venturing into the future. The rapid warming predicted for the coming decades is outside the range of historical experi-

ence. It is possible that both the climate and economic systems will exhibit at least some interactions not included in current calculations. A prime candidate on the climate side is the possible variation of cloud optical thickness as climate warms, which can greatly affect the degree of warming. On the economic side, the relationship between electrical demand and temperature might change, especially if temperatures reach levels not previously experienced.

To compare the climate model's sensitivity to that of the real world, two approaches have been used. One is to estimate how temperature has changed over the past century, and then to compare that (relatively small) change to the strength of the probable climate forcing mechanisms<sup>7</sup>. This procedure has led to the view that climate models can predict future global temperature changes from a doubling of atmospheric  $\text{CO}_2$  to within about a factor of two. It cannot yet provide a more precise evaluation because of uncertainties in our understanding of ocean heat uptake. This same uncertainty prevents us from firmly establishing the rate of future climate change. The second approach, used in assessing the climate model's suitability for evaluating large climate changes, is through the simulation of palaeoclimates, such as the last Ice Age. Although the models have produced the proper degree of cooling in the extratropics, the results are strongly constrained by the Ice Age boundary conditions of land-ice and sea-surface temperatures which are used as inputs. Even under these conditions, there are first-order uncertainties in the amount of cooling at low latitudes<sup>8</sup>. It is not known whether the models can realistically portray the entrance into or exit from an Ice Age, because the very long time integrations required (thousands of years) make the project unfeasible. Furthermore, climate models lack the necessary glacial dynamics.

Thus, confidence in our ability to model large climate changes (in the order of the  $4^\circ\text{C}$  global cooling of the Ice Age or the comparable warming of an atmosphere with a doubled level of  $\text{CO}_2$ ) lies in our assessment of the approximate climate sensitivity, and in our expectation (or hope) that we understand the physical principles which dominate within the expected range of mean temperatures. Of more dubious quality are regional and local forecasts of climate change, especially forecasts of the changes in the hydrological cycle (precipitation, soil moisture and so on) which depend upon the crudely modelled physics of convection and ground hydrology. Nevertheless, for climate models, there is widespread conviction that the basic uncertainties are solvable, so that models and predictions should improve with time.

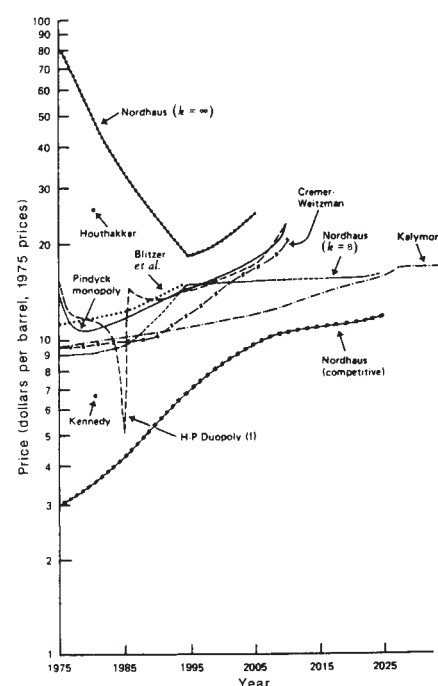


Fig. 2 Estimates of the time path of the price of oil, made between 1975 and 1977, from economic models with differing assumptions about OPEC pricing policies<sup>11</sup>. The pricing policies include maximization of monopoly profits, maximization of the cartel's total revenue, and maintenance of members' production shares. The bottom curve estimates the competitive market price in the absence of a cartel. The figure illustrates the uncertainty in economic forecasts due to unpredictable policy behaviour, as well as the ability of economic models to simulate a range of alternative scenarios.

Economic modellers did not predict the rapid rise in oil prices during the 1970s and thus its global consequences. Yet this failure arose not because the natural resource was limited, which could have been predicted, but from unforeseen political decisions. In fact, OPEC policy-making continues to create uncertainty about the future path of energy costs (see Fig. 2). This example highlights what can and cannot be expected from economic forecasting models. Characteristics of the economic system which are already in evidence, such as the distribution of natural resources or well-established behavioural tendencies, may be expected to influence economies in the future and their reaction to climate change. Long-term forecasting models should be able to calculate potential interactions among these components. Political decisions, however, will remain unpredictable, although the direction and relative scale of the effects of different policies can be estimated.

All economic decisions are made in a technological context. A given state of technology allows economic agents a specific range of choices. This increases the difficulty of predicting average behaviour, but defines the extent of possible variations. Over time, however,

technology itself is subject to change, as society adjusts to limitations on the quantity and use of resources by inventing new resources or new ways to use existing resources more efficiently. Technological change has become such an expected response to scarcity or potential gains that many economic models embody some form of induced innovation in their equations. However, very few such models actually provide a framework for defining and analysing alternative technologies of the future.

### Absence of links

One of the main problems with the joint application of climate and economic models is the absence of links, that is, processes and variables common to both types of model. Although the forecasting models run by crop producers or electrical utilities contain climate variables, most policy-orientated models do not. To some extent this is because data are often not available on the sensitivity of economic processes to climate. The information that does exist usually relates to extreme weather events, such as droughts or floods, which are followed by a return to normal conditions. As climate change represents a long-term shift away from the mean, and no such shift has occurred during the modern technological era, there simply aren't any relevant data.

Physical process models are now being used as intermediate steps between climate and economic models. These models, such as dynamic process crop growth models and watershed hydrological models, estimate the effects of climate changes on variables of economic interest, such as wheat yield or water availability. Changes in these variables generated by the process models are then used as inputs in economic models. The physical process models are useful because they provide detailed responses of the modelled system to climate change, but they are cumbersome as linkages because they must be rerun for each possible scenario. Another problem is that they require climatic variables on scales smaller than the climate model's resolved grid. Process models are available for only certain aspects of the economy, particularly agriculture, forestry and water resources. Models of other potentially sensitive processes, such as demographic response to climate change, do not exist because of lack of data.

Furthermore, without the use of aggregated macroeconomic models, the full scale of economic interaction and consequence cannot be evaluated. The effects of climate change will probably ricochet through the economy, and assessments based on keeping most aspects constant are unlikely to be correct in the long run. The situation is very similar to that of the climate analysis itself: the feedbacks of the physical system provide the greater part of

the warming effect of increased CO<sub>2</sub> on climate.

Given the current status of the different models and their linkages, what are the improvements needed to increase our ability to evaluate the economic effects of climate change? Climate modellers must improve regional forecast accuracy, by including more realistic ocean and ground hydrology along with improved parameterizations of clouds and convection in their models. Better ocean models will require many additional observations of ocean processes, and funding agencies must provide support for group ocean-modelling efforts.

Economic models must include explicit climate-sensitive functions. Intermediate physical process models, when appropriate, should generate response surfaces to specify these relationships. Additional research on the often subtle dependence of human society on climate is needed.

There is a need to design macro-economic models specifically to assess the aggregate economic effect of global climate change. For this purpose economists should adopt a customized approach to aggregating markets and groups of economic agents, explicitly including utilities and other activities such as agriculture, forestry, fishing and recreation which are known to respond directly to climate variables.

The spatial mismatch between geometric grids in climate models and geopolitical grids in economic models must be overcome. We need to generate geographical databases of economic information (for example urban, rural, industrial and agricultural identifiers) that are consistent with the gridbox resolutions of climate models, while recognizing the transport mechanisms that disperse the product throughout the economy. Physical process models should be developed for larger scales, consistent with the resolution of the climate model. And climate modellers should attempt to use as fine a spatial resolution as is practical. This would provide results on more appropriate spatial scales, which one could then aggregate to maintain the integrity of geopolitical units.

Improving the linkages will require interdisciplinary research by physical scientists and economists, as has been done in studies of energy and oil markets. In the academic world, one department ideally suited to the purpose is geography, which overlaps both physical and social sciences. In this regard, the recent trend towards phasing out geography departments at Ivy League universities may well be short-sighted.

For time-scales extending into the middle of the next century, the relationships between the economy (particularly greenhouse gas release) and climate will be interactive. At present, the only pos-

sible way to incorporate this relationship in studies of climate change is to iterate solutions of the separate models. It would be useful to start work upon a global climate-economic model. Given the flexibility of economic models, the best approach would be to build an economic model into the framework of the climate model. The resulting joint model could be employed at this stage to explore the basic dynamic properties of the systems and to establish ranges of parameters. It could then be determined which economic sectors are most sensitive to climate change and thus need to be modelled in greater detail.

In the foreseeable future, we are unlikely to be able to develop models to predict extreme events such as calamities of last October or the effect of the OPEC oil boycott on oil prices in the 1970s. Even prediction of the long-term changes may be beyond our ability, if unforeseen extreme events (such as war) occur. Thus programmes devoted to research into the economic effects of climate change should not be expected to provide bottom-line assessments of cost. The proper use of such models is to run alternative simulations and investigate potential magnitudes of total costs, relative gains and losses, and possible courses of action to minimize disruption or maximize opportunity.

### Frank assessment

A frank assessment of the (lack of) speed with which progress in this area is occurring leads to the conclusion that it is very possible that a dramatic change in climate will arrive before any adequate assessments of its economic effects become available. To make matters worse, climate change will be a continuing phenomenon, so adjustments made in one decade may well be inappropriate in the next. There is a great deal of work to be done by modellers of both persuasions if we are to be in a position to prepare for, instead of simply react to, climate change. □

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# Solar neutrinos: a field in transition

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*Solar-neutrino experiments provide a unique opportunity for studying weak interactions in a realm where new physics may be revealed. At the same time the neutrinos carry important information about the physical processes occurring in the otherwise inaccessible core of a main-sequence star. A variety of experiments, electronic and radiochemical, must be carried out to determine what new physics and astronomy are being uncovered with these underground detectors.*

SOLAR-neutrino experiments that are in progress will soon decide whether the long-standing puzzle known as the 'solar neutrino problem' requires new physics or new astronomy. For about two decades, a solar-neutrino experiment that uses <sup>37</sup>Cl has been in conflict with predictions made with the best calculations of the standard-solar model and the assumption that neutrinos reach us from the interior of the Sun without being affected in transit. This result has been confirmed in the past year in the form of a limit on the <sup>8</sup>B flux by using a large water detector to detect neutrino-electron scattering. Particle physicists have recently also realized that there are theoretically attractive solutions to the solar neutrino problem which are natural in some grand unified theories. In this article, we review the present state of both the theory and the experiments.

Table 1 lists the experiments, planned or in progress, that we shall discuss in this review.

## Neutrino physics

The existence of the neutrino was first suggested by Pauli to explain the apparent missing energy in nuclear  $\beta$ -decay. The basic  $\beta$ -decay processes are

$$n \rightarrow p + e^- + \bar{\nu}_e \quad (1a)$$

$$p \rightarrow n + e^+ + \nu_e \quad (1b)$$

The proton transformation, equation (1b), is involved in the processes producing neutrinos in the Sun such as reactions 1 and 9 in Table 2. The standard methods for detecting neutrinos involve 'inverse beta' reactions, for which the basic processes are

$$\bar{\nu}_e + p \rightarrow n + e^+ \quad (2a)$$

$$\nu_e + n \rightarrow p + e^- \quad (2b)$$

and neutrino-electron scattering,

$$\nu_e + e \rightarrow \nu_e' + e' \quad (2c)$$

The transformation shown in equation (2b), occurring inside the <sup>37</sup>Cl nucleus and converting it to <sup>37</sup>Ar, is the basis of the original solar-neutrino experiment that is discussed below. Equation (2c) has recently been used to set a confirmatory limit on the solar neutrino flux and, with the same detector, equation (2a) was used to observe antineutrinos from the supernova in the Large Magellanic Cloud.

There are two other types of neutrinos,  $\nu_\mu$  and  $\nu_\tau$ , that are distinguished from  $\nu_e$  by the way they interact with matter. The analogue of the inverse beta reaction for  $\nu_\mu$  would be a reaction in which a muon was produced instead of an electron. Such reactions are energetically impossible for neutrinos with energies <106 MeV; therefore reactions such as neutrino capture on <sup>37</sup>Cl

Table 1 Solar neutrino experiments

| Target                  | Threshold (MeV) | Source(s)                        | Group(s)                   |
|-------------------------|-----------------|----------------------------------|----------------------------|
| <sup>37</sup> Cl        | 0.8             | <sup>8</sup> B( <sup>7</sup> Be) | Pennsylvania               |
| $\nu_e$ -e <sup>-</sup> | 5-10            | <sup>8</sup> B                   | Kamiokande II              |
|                         |                 |                                  | LVD                        |
|                         |                 |                                  | University of Sydney       |
|                         |                 |                                  | Canada/USA/England         |
|                         |                 |                                  | Icarus                     |
| <sup>98</sup> Mo        | >2              | <sup>8</sup> B                   | LANL                       |
| <sup>2</sup> H          | ~5              | <sup>8</sup> B                   | Canada/USA                 |
| <sup>40</sup> A         | ~5              | <sup>8</sup> B                   | Icarus                     |
| <sup>81</sup> Br        | 0.5             | <sup>8</sup> B, <sup>7</sup> Be  | Pennsylvania and Tennessee |
| <sup>71</sup> Ga        | 0.2             | pp( <sup>7</sup> Be)             | GALLEX; USSR               |
| <sup>115</sup> In       | 0.1             | pp                               | British; French            |
| <sup>11</sup> B         | ≤2              | <sup>8</sup> B                   | Bell Laboratories          |
| <sup>4</sup> He         | <0.1            | pp                               | Brown University           |

could not detect  $\nu_\mu$  nor, also for energetic reasons,  $\nu_\tau$ . On the other hand, the elastic scattering of neutrinos from electrons (equation (2c)) can be used to detect all types of neutrinos, but the scattering cross section is seven times larger for  $\nu_e$  than for  $\nu_\mu$  or  $\nu_\tau$ .

No conclusive evidence exists that neutrinos have a mass. Direct experiments<sup>1</sup>, such as the measurement of the electron energy spectrum from <sup>3</sup>H decay, have yielded only upper limits on the masses. These are  $m(\nu_e) < 20$  eV,  $m(\nu_\mu) < 300$  keV, and  $m(\nu_\tau) < 70$  MeV. There are arguments<sup>2</sup> based upon cosmology that strongly suggest that none of the neutrinos has a mass >80 eV.

The standard (Weinberg-Salam-Glashow) model of weak interactions is normally presented in a form in which neutrinos are massless. But most extensions of the model give the neutrinos a mass. Of particular interest are grand unified theories (GUTs), which unify the different types of interactions (weak, electromagnetic and strong) into a single interaction at very high energies or equivalently at a very large mass scale,  $M$ . The simplest of these theories, called SU(5), does not require a neutrino mass; however, this theory appears to be ruled out by the failure to observe the decay of the proton. Most other GUTs, exemplified by one called SO(10), require that neutrinos have a mass. While these theories also, in general, predict proton decay, the theoretical rate is uncertain and so they cannot be ruled out on the basis of existing experiments.

In GUTs, the neutrino masses are inversely proportional to the scale  $M$ . For  $M \sim 10^{15}$  GeV, as required in many GUTs, all

neutrinos have masses  $\ll 1$  eV. Furthermore, one expects  $m(\nu_e) \ll m(\nu_\mu) \ll m(\nu_\tau)$  so that all masses are many orders of magnitude smaller than the range accessible by direct experiments. Theories of neutrino mass are summarized in recent reviews<sup>3,4</sup>.

It is possible to explore much smaller neutrino masses by the indirect method of neutrino oscillations. Theories of neutrino mass strongly suggest that the neutrino  $\nu_e$  (or  $\bar{\nu}_e$ ) emitted in the  $\beta$ -decay process does not have a well-defined mass but rather is a quantum-mechanical coherent mixture of mass eigenstates. In most theories the mixture making up  $\nu_e$  is primarily one mass eigenstate  $\nu_1$ , the lightest of the three, and the one that would be measured in direct experiments. (For two neutrinos,  $\nu_e$  and  $\nu_\mu$ , one can write  $\nu_1 = \nu_e \cos \Theta - \nu_\mu \sin \Theta$ ,  $\nu_2 = \nu_e \sin \Theta + \nu_\mu \cos \Theta$ , where  $\nu_1, \nu_2$  are the mass eigenstates and  $\Theta$  is the mixing angle.)

The small admixture (in typical theories of the order of 0.2 in the amplitude) of other mass eigenstates can lead to the striking phenomenon of neutrino oscillation. As the neutrino propagates through the vacuum the relative phases of the different mass eigenstates change as a result of the mass differences. After some distance, the state that was originally  $\nu_e$  is found to be a mixture of  $\nu_e$  with  $\nu_\mu$  or  $\nu_\tau$ . This is a quantum-mechanical effect analogous to the quantum-mechanical treatment of the precession of a spin in a magnetic field. The distance,  $l_\nu$ , required for one complete oscillation from  $\nu_e$  to  $\nu_\mu$  and back to  $\nu_e$  in vacuum is given by

$$l_\nu = (2.5 \text{ m}) E_\nu / \Delta m^2 \quad (3a)$$

where  $E_\nu$  is the neutrino energy in MeV, and  $\Delta m^2 = m_2^2 - m_1^2$  in  $\text{eV}^2$  for the case of two mass eigenstates  $\nu_1$  and  $\nu_2$  that are mixed. The probability  $P(\nu_e \rightarrow \nu_\mu)$  that a neutrino  $\nu_e$  becomes a  $\nu_\mu$  after travelling a distance  $D$  depends also on the mixing angle  $\Theta$ . For this case one has

$$P(\nu_e \rightarrow \nu_\mu) = \sin^2 \Theta \sin^2 (\pi D / l_\nu) \quad (3b)$$

Many experiments have been carried out searching for oscillations using neutrinos from reactors (MeV range) or accelerators (GeV range). These experiments yield limits on neutrino mass differences depending on the assumed amounts of mixing. For the amounts of mixing suggested by most GUTs and with  $m(\nu_1) \ll m(\nu_2)$ , these experiments<sup>1</sup> place an upper limit of between 0.1 and 1 eV on  $m(\nu_2)$ , which corresponds essentially to a limit on  $m(\nu_\mu)$ . Because  $l_\nu$  increases as the mass difference decreases it is not possible to probe much smaller neutrino masses in laboratory experiments.

Solar neutrinos are sensitive to oscillations corresponding to much smaller neutrino masses because they travel astronomically large distances as they propagate from the centre of the Sun to the detector on Earth. Experiments with solar neutrinos can extend the range of sensitivity down to

$$\Delta m^2 \geq 10^{-11} \text{ eV}^2. \quad (4)$$

Thus the study of solar neutrinos provides a natural method of extending the study of neutrino oscillations to the tiny mass differences that are natural in GUTs.

## The standard solar model

The 'standard solar model' is the theoretical model that is used to interpret solar neutrino experiments. The standard solar model is constructed from the most refined physical calculations and nuclear and solar data available at the time the model is calculated. Thus the set of numbers that correspond to the standard solar model vary with time<sup>5,6</sup>, hopefully (nearly) always getting closer to the 'true' standard model. Over the past five years, hundreds of experiments by nuclear physicists and by astronomers, as well as improved theoretical calculations of some of the input physics, have made possible a detailed study of the uncertainties as well as the best values for quantities predicted by the standard solar model. The reader will recognize

the enormous community effort that is embodied in this project: each one of, for example, the many important nuclear cross section measurements that were made during this period typically took a team of experts two or more years to complete and analyse the precise data. Bahcall and Ulrich have recently completed a systematic reinvestigation of the characteristics and uncertainties of the standard model<sup>6</sup>; here we shall use the results of that study in describing the theoretical expectations. The net result of all the changes is a standard model that looks similar to that of the previous systematic study<sup>7</sup> with predicted neutrino fluxes that, somewhat by chance, are almost unchanged.

Some of the principal approximations used in constructing standard models deserve special attention: because of their basic nature they have been investigated particularly thoroughly or often for possible sources of departure from the standard model.

(1) Hydrostatic equilibrium. The Sun is assumed to be in hydrostatic equilibrium; the radiative and particle pressures of the model exactly balance gravity. Observationally, this is known to be an excellent approximation because a gross departure from hydrostatic equilibrium would cause the Sun to collapse in a free fall time, which is less than an hour.

(2) Energy transport by photons or convective currents. In the deep interior, which is most important for the solar neutrino problem, the energy transport is primarily by photon diffusion; the calculated radiative opacity is a crucial ingredient in the construction of a model and has been the subject of a number of recent detailed studies.

(3) Energy generation by nuclear reactions. The primary energy source for the radiated photons and neutrinos is nuclear fusion, although the small effects of gravitational contraction (or expansion) are included.

(4) Abundance changes caused solely by nuclear reactions. The primordial solar interior is presumed to have been chemically homogeneous. Changes in the local abundances of individual isotopes occur only by nuclear reactions in those regions of the model that are convectively stable.

A standard solar model is really a sequence of models. One begins with a main-sequence star that has a homogeneous composition. Hydrogen burns in the core of the model, supplying both the radiated luminosity and the local heat (thermal pressure) that supports the star against gravitational collapse. Successive models are calculated by allowing for composition changes caused by nuclear reactions, as well as the mild evolution of other parameters. The later models in an evolutionary sequence have inhomogeneous compositions, the innermost mass fraction of hydrogen being about one-half the surface (primordial) value. Typically, an evolutionary sequence requires

**Table 2** Reactions

| Reaction                                                         | No. | Terminations (%) | Neutrino energy (MeV)      |
|------------------------------------------------------------------|-----|------------------|----------------------------|
| $p+p \rightarrow {}^2\text{H} + e^+ + \nu_e$                     | 1a  | 99.75            | $\leq 0.420$               |
| or                                                               |     |                  |                            |
| $p+e+p \rightarrow {}^2\text{H} + \nu_e$                         | 1b  | 0.5              | 1.442                      |
| ${}^2\text{H}+p \rightarrow {}^3\text{He} + \gamma$              | 2   | 100              | —                          |
| ${}^3\text{He}+p \rightarrow {}^4\text{He} + e^+ + \nu_e$        | 3   | 0.00002          | $\leq 18.77$               |
| or                                                               |     |                  |                            |
| ${}^3\text{He}+{}^3\text{He} \rightarrow \alpha + 2p$            | 4   | 85               | —                          |
| or                                                               |     |                  |                            |
| ${}^3\text{He}+{}^4\text{He} \rightarrow {}^7\text{Be} + \gamma$ | 5   | 15               | —                          |
| ${}^7\text{Be}+e^- \rightarrow {}^7\text{Li} + \nu_e$            | 6   | 15               | 0.861 (90%)<br>0.383 (10%) |
| ${}^7\text{Li}+p \rightarrow 2\alpha$                            | 7   | —                | —                          |
| or                                                               |     |                  |                            |
| ${}^7\text{Be}+p \rightarrow {}^8\text{B} + \gamma$              | 8   | 0.02             | —                          |
| ${}^8\text{B} \rightarrow {}^8\text{Be}^* + e^+ + \nu_e$         | 9   | —                | $< 15$                     |
| ${}^8\text{Be}^* \rightarrow 2\alpha$                            | 10  | —                | —                          |



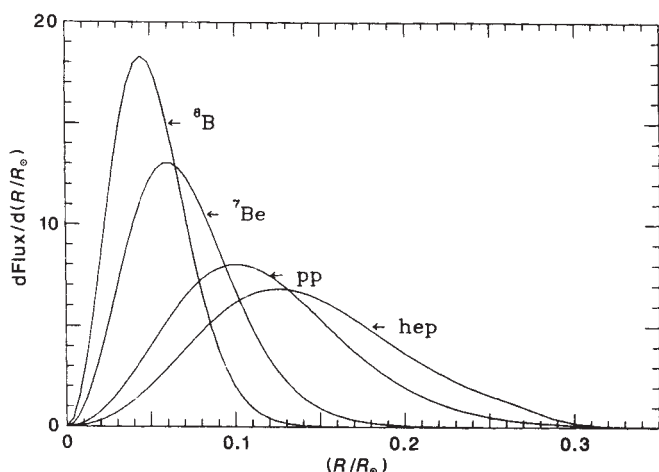


Fig. 1 Neutrino production as a function of radius. The fraction of neutrinos that originate in each fraction of the solar radius is  $[d\text{Flux}/d(R/R_\odot)]/[d(R/R_\odot)]$ . The figure from ref. 6 illustrates the production fraction for  $^8\text{B}$ ,  $^7\text{Be}$ , pp, and hep neutrinos for the standard solar model.

about five to seven solar models of progressively greater ages to match the luminosity and radius to the desired one part in  $10^5$ .

Evolutionary codes have recently been developed by a number of different groups; when adjusted for differences in input parameters, all the codes yield solar neutrino fluxes that are in agreement with each other to  $\sim 10\%$  or better.

Constructing a solar model is an eigenvalue problem, with fixed boundary conditions in both space and time. We seek a model with a total luminosity (in photons) equal to  $L_{\text{solar}}$  and an outer radius  $R_{\text{solar}}$  at an elapsed time of 4,600 Myr. The initially assumed values of chemical composition and entropy are iterated until an eigenvalue is obtained. The luminosity boundary condition has an especially strong effect on the calculated neutrino fluxes. The reason is that both the luminosity and the neutrino fluxes are produced by nuclear reactions in the solar core. The strong coupling between the luminosity and the neutrino fluxes has resulted in some groups deriving incorrect results because they have not iterated the models sufficiently.

An eigenvalue solution determines the primordial values for the mass fractions of hydrogen, helium and heavy elements, the present complete run of physical variables inside the Sun, the spectrum of helioseismology oscillation frequencies observed on the surface of the Sun, and, of course, the neutrino fluxes.

The Sun shines by converting four protons to an  $\alpha$ -particle, two positrons and two neutrinos. The excess mass released by this nuclear transformation is about 25 MeV, most of which is deposited in the star as thermal energy that ultimately leaks out from the surface as visible photons.

Table 2 lists the principal nuclear reactions that are believed to be most important in converting protons to  $\alpha$ -particles in the solar interior; the chain of reactions in Table 2 is known as the pp chain (after the initial reaction). The second column of Table 2 gives the percentage of terminations of the pp chain (conversion of four protons to an  $\alpha$ -particle) in which the indicated reaction occurs. Thus essentially all the reactions begin with a slow pp reaction; only  $\frac{1}{4}\%$  start with an electron being captured by two protons and producing a 1.4-MeV neutrino. The last column of Table 2 gives the neutrino energy released by each nuclear reaction.

The dominant neutrino source in both the  $^{37}\text{Cl}$  and the Kamiokande II experiments is the rare but high-energy  $^8\text{B}$  neutrinos (reaction 9), produced only about twice in every  $10^4$  terminations of the pp chain. The  $^7\text{Be}$  neutrinos (reaction 6) are

expected to contribute about 15% to the  $^{37}\text{Cl}$  experiment; an other sources contribute much less on the basis of the standard solar and particle physics models. The energy threshold and sensitivity of both the  $^{37}\text{Cl}$  and the Kamiokande experiments discriminate strongly against the other, more abundant neutrino sources.

Reaction 4 is extremely rare and is unimportant for all radiochemical experiments suggested so far. But the high energy of the  $^3\text{He} + p$  neutrinos, hereafter hep neutrinos, makes their detection possible and significant in large next-generation experiments to measure the energies of individual electrons that participate in neutrino events.

Figure 1 shows where the energy production and the neutrino fluxes originate in the standard solar model. The energy generation peaks at a radius of  $0.09 R_\odot$ , which corresponds to about  $0.06 M_\odot$ ; the half-peak values of the energy generation extend from  $0.04 R_\odot$  to  $0.16 R_\odot$ , that is, from the inner  $0.007 M_\odot$  to  $0.23 M_\odot$ . The peak of the energy generation occurs at a temperature of  $\sim 1.4 \times 10^7$  K and a density of  $\sim 95 \text{ g cm}^{-3}$ . The region in which the pp flux is produced is similar to that of the total energy generation. Because of its strong temperature dependence, the  $^8\text{B}$  production peaks at much smaller radii,  $0.05 R_\odot$  and  $T = 1.5 \times 10^7$  K, and is generated in a narrower region,  $0.02 R_\odot$  to  $0.07 R_\odot$ . The region of  $^7\text{Be}$  production is intermediate between the  $^8\text{B}$  and pp regions, peaking at  $0.06 R_\odot$  and spreading (half-peak range) from  $0.03 R_\odot$  to  $0.10 R_\odot$ . The hep distribution is the most extended, ranging (half-peak points) from  $0.06 R_\odot$  to  $0.21 R_\odot$  with a peak at  $0.13 R_\odot$  and  $T = 1.2 \times 10^7$  K.

Table 3 gives the best estimates for each of the neutrino fluxes, together with the theoretical uncertainties. The fluxes were computed for the standard solar model<sup>6</sup>.

The 'solar neutrino problem' is the difference between the observed rate of neutrino events in the  $^{37}\text{Cl}$  (refs 8–10) and Kamiokande II experiments<sup>11–15</sup> on the one hand and the rate calculated from the standard solar model and the standard electroweak model (of particle physics) on the other hand. It is therefore crucial to know the uncertainties in the predictions of the standard solar model. The procedure that has been adopted<sup>6</sup> is to quote a 'total theoretical range' which corresponds to  $3\sigma$  errors for experimentally measured quantities and the total range

Table 3 Neutrino fluxes

| Source          | Flux ( $10^{10} \text{ cm}^{-2} \text{ s}^{-1}$ ) |
|-----------------|---------------------------------------------------|
| pp              | 6.0 ( $1 \pm 0.02$ )                              |
| pep             | 0.014 ( $1 \pm 0.05$ )                            |
| hep             | $8 \times 10^{-7}$ ( $1 \pm ?$ )                  |
| $^7\text{Be}$   | 0.47 ( $1 \pm 0.15$ )                             |
| $^8\text{B}$    | $5.8 \times 10^{-4}$ ( $1 \pm 0.37$ )             |
| $^{13}\text{N}$ | 0.06 ( $1 \pm 0.50$ )                             |
| $^{15}\text{O}$ | 0.05 ( $1 \pm 0.58$ )                             |
| $^{17}\text{F}$ | $5.2 \times 10^{-4}$ ( $1 \pm 0.46$ )             |

of plausible theoretical estimates for quantities that must be calculated. The error estimates given in Table 3 for the neutrino fluxes correspond to this definition of 'total theoretical range'.

The solar-neutrino experiments that measure individual recoil electrons (like the natural water electron scattering detectors and the  $^2\text{H}$  and  $^{40}\text{Ar}$  experiments discussed below) sample neutrinos from two different parts of the solar interior. These detectors are sensitive to both the  $^8\text{B}$  neutrinos, produced at relatively small radii and high temperatures, and the hep neutrinos, which are generated at larger radii and lower temperatures. Fortunately, it is possible to identify the highest energy recoil electrons as coming from events initiated by hep neutrinos, allowing the same experiment to probe regions of the solar interior that are both inside (the dominant  $^8\text{B}$  neutrinos) and outside (the hep neutrino) the main energy-producing region (Fig. 1).

The flux of neutrinos from the proton-proton reaction is the most accurately known; the estimated uncertainty in this flux is only 2%. It is often mistakenly said in the literature that the flux of this reaction is fixed by the observed solar luminosity. In fact the computed flux of pp solar neutrinos would be about one half as large if  ${}^3\text{He}$  were burned primarily by interactions with an  $\alpha$ -particle rather than (as in the standard model) by interactions with other  ${}^3\text{He}$  nuclei. Two pp reactions are required to terminate the chain through reaction 4 of Table 2 (which predominates in the standard solar model), whereas only one pp reaction is necessary if reaction 5 dominates. Fortunately, the cross section factors for reactions 4 and 5 are relatively well known (see Table 2); they indicate that reaction 4 occurs about six times as often as reaction 5 under average solar conditions.

The uncertainty in the flux of  ${}^7\text{Be}$  neutrinos is also moderately small,  $\sim 14\%$ .

The uncertainty in the crucial  ${}^8\text{B}$  neutrinos is large, 34%. The greatest uncertainties in the calculation of this flux derive from the measurement of the low-energy nuclear cross section factor for the production of  ${}^8\text{B}$  and from the primordial abundance of heavy elements (estimated from the currently observed surface abundances). The uncertainties are even larger,  $\sim 40\text{--}50\%$ , for neutrinos from the carbon-nitrogen-oxygen (CNO) nuclear burning cycle. The biggest contributors to their uncertainties are the heavy-element abundance and the rate at which  ${}^{14}\text{N}$  is burned. An additional nuclear physics measurement must be performed before one can estimate reliably the uncertainty in the rare but important hep neutrinos.

## The solar neutrino problem

The  ${}^{37}\text{Cl}$  detector in the Homestake Mine, Kellogg, South Dakota (the largest operating gold mine in the United States) was for two decades the only operating neutrino experiment with sufficient sensitivity to observe the solar neutrino flux. Since the initial observations in 1968, it has become clear that the solar-neutrino capture rate in  ${}^{37}\text{Cl}$  is below theoretical expectations. R. Davis, Jr, B. Cleveland and J. K. Rowley have reported<sup>8-10</sup> on the results of the measurements performed over the period 1970-85. The average neutrino capture rate of this period was  $2.07 \pm 0.25$  SNU ( $1\sigma$  error), a result that conflicts with the standard model prediction<sup>6</sup> of  $7.9 (1 \pm 0.33)$  SNU (total theoretical range) (here SNU = solar neutrino unit  $= 10^{-36}$  interactions  $\text{s}^{-1}$  per atom). This discrepancy between standard theory and observation has persisted for almost two decades and has become known as 'the solar neutrino problem'.

There are two types of 'solution' to the solar neutrino problem, those that modify the physics of the Sun, yielding a non-standard model, and those based on assumptions about properties of neutrinos.

Non-standard solar models are constructed by changing something, physics or input data, from our current understanding to something less plausible<sup>16-20</sup>. They include models with enhanced element diffusion, postulated convective instabilities, a massive black hole in the solar interior, and a solar interior deficient in heavy elements. Most of the published non-standard models were invented to reduce the central temperature of the Sun and thus severely reduce the flux of  ${}^8\text{B}$  neutrinos.

One suggestion has been made<sup>21-23</sup> that can solve simultaneously the famous 'missing mass' problem in astronomy and the solar neutrino problem. In this suggestion, weakly interacting massive particles (WIMPs) are produced in the early Universe in an abundance that accounts for the unseen matter in large astronomical systems. The properties of the so-far undiscovered WIMPs could be just such that they carry some of the heat out of the solar interior, smoothing out the temperature gradient and reducing the flux of  ${}^8\text{B}$  neutrinos to be consistent with the experimental limit.

In the other class of solutions the flux of neutrinos at the source in the centre of the Sun is given by the standard solar model but these neutrinos are transformed from  $\nu_e$  to another

type on their journey to the detector. For nearly all proposed solutions this transformation is related to neutrino mass.

One of the oldest suggestions<sup>24,25</sup> is that  $\nu_e$  oscillates to  $\nu_\mu$  or  $\nu_\tau$  in the vacuum between the Sun and the Earth. Values of  $\Delta m^2 \geq 10^{-10} \text{ eV}^2$  correspond to oscillation lengths equal or smaller than the Earth-Sun distance for the most easily observable high-energy solar neutrinos. Thus if  $m(\nu_e) \ll m(\nu_\mu)$  a value of  $m(\nu_\mu)$  as small as  $10^{-5} \text{ eV}$  would be sufficient. But to get the large suppression observed it is necessary to assume that  $\nu_e$  is an almost equal mixture of the different mass eigenstates, which is not expected in GUTs.

If  $\nu_e$  is not the lightest of the neutrinos, a possible solution would be the decay of  $\nu_e$  to a lighter neutrino plus a photon or a scalar boson. But the observation that  $\bar{\nu}_e$  travelled the much larger distance from the supernova SN 1987A rules this out unless there is a large amount of mixing in addition to decay<sup>26</sup>.

A massive Dirac neutrino may have a magnetic moment. If this moment were as large as  $10^{-10}$  to  $10^{-11} \mu_B$  (Bohr magnetons), the left-handed  $\nu_e$  could have its spin flipped to a right-handed  $\nu_e$  in passing through magnetic fields of a few kG in the outer part of the Sun<sup>27</sup>. Right-handed neutrinos do not have normal weak interactions and would therefore not be detected. This solution has recently been revived<sup>27-29</sup> to account for an apparent anti-correlation of the neutrino capture rate with the sunspot cycle<sup>9</sup>. The greatest change occurred at the time of the onset of solar cycle 21 in 1977-80. The large magnetic moment needed for this solution, although consistent with present empirical limits<sup>30</sup> of the order of  $10^{-10} \mu_B$ , is considered unlikely from a theoretical point of view<sup>31</sup> by most authors (but see refs 32, 33). An alternative explanation of the apparent correlation is that it is due to a rather improbable statistical fluke<sup>34,35</sup>. Observations of the neutrino capture rate with the  ${}^{37}\text{Cl}$  experiment in 1990 and 1991 will help to resolve this question.

A solution consistent with the GUT theories of neutrino mass was proposed three years ago<sup>36</sup>. This involves the conversion of  $\nu_e$  into  $\nu_\mu$  or  $\nu_\tau$  as the neutrino passes through the material medium of the Sun. This conversion can be viewed as an oscillation that is enhanced by the effect of the medium on the propagation phase of the neutrinos. It is usually referred to as the Mikhaevyev-Smirnov-Wolfenstein (MSW) effect and is discussed in more detail below.

There are some fundamental differences between solutions based on non-standard solar models and those based on neutrino mass. In non-standard solar models the shape of the neutrino spectrum from individual neutrino sources, such as the  ${}^8\text{B}$  spectrum, will be the same as in Fig. 1. Only the total flux from a given source can be altered by solar physics; the shape is fixed by nuclear physics. In contrast the transformation of  $\nu_e$  to  $\nu_\mu$  or  $\nu_\tau$  by oscillations is energy dependent and so the spectrum in general is distorted. Nearly all proposed non-standard solar models predict the same flux as the standard model for the predominant  $\nu_e$  from the pp reaction 1a. In contrast, it is possible as a result of neutrino transformation processes to decrease the flux of those neutrinos as well as those from  ${}^8\text{B}$  decay.

In oscillation models the missing  $\nu_e$  arrive as  $\nu_\mu$  or  $\nu_\tau$ . Thus detectors based on neutrino-electron scattering should be able to detect the missing neutrinos. For example, in the extreme case that all  $\nu_e$  of a certain energy are transformed to  $\nu_\mu$  or  $\nu_\tau$ , the neutrino-electron scattering rate is reduced to about one seventh of the standard prediction. It is, however, possible to imagine theories in which the oscillations take  $\nu_e$  into a new type of non-interacting, or sterile, neutrino<sup>3</sup> so that there would be no way to detect them.

## The MSW effect

Neutrino oscillations occur because of the variation in the relative phases of different components of the neutrino wave function with time or distance. In vacuum this variation is entirely due to the different masses and consequent different energies of the mass eigenstates. In a material medium there is



an additional effect<sup>37</sup> because of the index of refraction of neutrinos which, by the optical theorem, is proportional to the density of scatterers times the forward scattering amplitude. In particular the phase of  $\nu_e$  varies relative to that of  $\nu_\mu$  and  $\nu_\tau$  because the elastic scattering of the  $\nu_e$  by electrons is larger. The electron neutrinos interact with electrons through both the charged and neutral currents, whereas  $\nu_\mu$  and  $\nu_\tau$  interact only by neutral currents. As a result neutrino oscillations are different in matter than in a vacuum.

The detailed analysis shows that for a sizeable range of neutrino parameters the neutrinos emerging from the solar interior will pass through a region where the electron density is such that the oscillation amplitude is maximized. This is called the MSW effect. In fact because the density is varying it is possible that instead of a true oscillation the original  $\nu_e$  will adiabatically transform into the state  $\nu_2$ , which is primarily  $\nu_\mu$  or  $\nu_\tau$ . The large suppression of the  $^8\text{B}$  flux observed in the  $^{37}\text{Cl}$  experiment can be explained if the parameters satisfy roughly<sup>36,38-40</sup>

$$\Delta m^2 \leq 10^{-4} \text{ eV}^2 \quad (5a)$$

$$(\sin^2 2\theta)\Delta m^2 \geq 3 \times 10^{-8} \text{ eV}^2 \quad (5b)$$

$$\sin^2 2\theta < 0.8 \quad (5c)$$

where  $\tan \theta$  is the relative amplitude of  $\nu_1$  and  $\nu_2$  in the  $\nu_e$  wave function. For a typical amount of mixing expected theoretically ( $\sin \theta \sim 0.2$ ) and  $m(\nu_2) \gg m(\nu_1)$ , this corresponds to a value of  $m(\nu_2)$  between  $5 \times 10^{-4}$  and  $10^{-2}$  eV. The range of values in equation (5a-c) appears quite natural in the theoretical framework of GUTs. In general in GUTs the heaviest neutrino,  $\nu_\tau$ , has a mass of order  $(100 \text{ GeV})^2/M$ , where  $M$  is a large mass scale. In the simplest version of SO(10),  $M \sim 10^{15}$  GeV, the unification scale. Thus with  $m(\nu_e) \ll m(\nu_\tau)$  we would have  $m(\nu_\tau) \sim 10^{-2}$  eV and  $\Delta m^2 \sim 10^{-4} \text{ eV}^2$  so that  $\nu_e$  would transform into  $\nu_\tau$  provided that the mixing angle  $\theta$  was not smaller than  $10^{-2}$ . In more recent versions<sup>41</sup> of SO(10),  $M$  is given by an intermediate mass scale of order  $10^{11}$ – $10^{12}$  GeV. In this case the mass of  $\nu_\tau$  is too large to affect the solar neutrino problem although it could be of great importance in cosmology; however, the mass of  $\nu_\mu$  could now be of the order of  $10^{-2}$  eV so that the solar  $\nu_e$  would transform into  $\nu_\mu$ .

The MSW effect is an attractive solution to the solar neutrino problem that can only be tested by carrying out further experiments on solar neutrinos.

## Kamiokande II

The Kamiokande II experiment<sup>11-15</sup> detects neutrino events via the Cherenkov light emitted by electrons that are scattered in the forward direction by the solar neutrinos. This is the first of several solar-neutrino experiments that are planned or in progress in which  $\nu_e - e$  scattering will be studied. For the higher-energy neutrinos ( $>5$  MeV, that is,  $^8\text{B}$  and hep neutrinos only) that can be detected by this process, the scattering provides additional information not available with a radiochemical detector. One can derive unique information about the incident neutrino spectrum (from measurements of the recoil energies of the scattered electrons), determine the direction from which the neutrinos arrive, and record the precise time of the events.

The Kamiokande experiment was originally designed as a 3-kt water Cherenkov detector to study proton decay<sup>11</sup>. In late 1984 improvements were begun to make possible the detection of the relatively low-energy events that are expected to be produced by solar neutrinos. This detector also provided the first-ever observation of neutrinos from a supernova<sup>12</sup>; the supernova neutrinos happen to have energies in about the same range as those expected from solar  $^8\text{B}$  neutrinos. Fortunately, an upgrade of Kamiokande II to a full-time solar-neutrino detector had been completed just a few months before the blast of neutrinos from the Large Magellanic Cloud reached the Earth.

The initial results from the Kamiokande II detector yield an upper limit at the 90% confidence level for the flux of  $^8\text{B}$  neutrinos of<sup>11</sup>  $\phi(^8\text{B}) \leq 3.2 \times 10^6 \text{ cm}^{-2} \text{ s}^{-1}$ . This upper limit is  $\sim 55\%$  of the value calculated with the best standard solar model<sup>6</sup> and is inconsistent, at the 90% confidence level, with the range of values allowed by the recognized uncertainties in the calculation of the flux (see Table 3). The upper limit quoted above results from 128 days of low-background data accumulated from December 1986 to May 1987. Reductions in the background rate that are in progress should lead to an improved limit, or a detection, of the solar neutrino  $^8\text{B}$  flux, perhaps in 1988. Unfortunately, the Kamiokande II detector is not large enough to permit a robust detection of the hep neutrinos if the standard solar model is correct.

The Kamiokande II result is of great importance because until now all of the observational results on solar neutrinos have come from a single experiment, using the  $^{37}\text{Cl}$  detector.

If the MSW effect is the correct explanation of the solar neutrino problem, then a moderate increase in sensitivity in the Kamiokande II detector should reveal a solar-neutrino signal. The minimum rate expected from complete conversion of electron neutrinos to muon or tau neutrinos is 14% of the standard model prediction<sup>42,43</sup>. Thus Kamiokande II should see a definite solar neutrino signal with an increase in sensitivity of no more than a factor of 3.8 of the sensitivity already obtained, provided that the MSW effect is the correct explanation and the particles to which the electron neutrinos are converted are not 'sterile'.

## A geochemical experiment

A geochemical experiment has been developed over the last three years at Los Alamos National Laboratory<sup>44,45</sup>. Neutrinos are detected through their absorption by molybdenum atoms that are shielded from cosmic rays by the covering of a deep mine. The absorption of neutrinos produces an unstable but long-lived isotope of technetium that would not be present in a steady-state situation in which there were no high-energy solar neutrinos. The method uses the neutrino capture reaction  $\nu_e + ^{98}\text{Mo} \rightarrow ^{98}\text{Tc}^* + e$ . Neutrino absorption occurs to a variety of excited states of technetium that can be populated only by the  $^8\text{B}$  (and much less importantly, hep) neutrinos.

The  $^{98}\text{Tc}$  has a mean life of 4.2 Myr, requiring a geological timescale to accumulate a sufficient number of atoms ( $N \approx 10^7$ ) to observe by an ultra-sensitive mass spectrometer. The experiment will test for the constancy of the flux of  $^8\text{B}$  neutrinos over the past several million years.

The experimentalists take advantage of the Henderson molybdenum mine, in which the ore is recovered at great depth (1,500–1,800 m). The backgrounds have been carefully evaluated and are believed to be sufficiently low to permit the observation of  $^{98}\text{Tc}$  production from solar neutrinos. The largest uncertainty in the predicted rate, about a factor of two, comes from the neutrino absorption cross section<sup>6,46</sup>. The Los Alamos group takes advantage of the mineral processing plant. The molybdenum minerals ( $\text{MoS}_2$ ) are roasted in air to obtain the molybdenum as the oxide. Tiny amounts of rhenium, a chemical homologue of technetium, are used to trace the technetium through the extraction process. The relatively volatile  $\text{ReO}_2$  and  $\text{TcO}_2$  from the roaster are found in the water scrubber and can be readily isolated.

A sample of technetium containing about  $10^7$  atoms (originally obtained from about 20 boxcars of ore) is now essentially ready for mass spectrometric analysis. The first results from this ingenious experiment should be available during 1989.

## Gallium detectors

Two major solar-neutrino experiments using  $^{71}\text{Ga}$  are under way, one by a primarily European collaboration (GALLEX)<sup>47,48</sup> and the second by a group which will work in the Soviet Union (Institute for Nuclear Research, Moscow)<sup>49,50</sup>. The GALLEX

collaboration will use 30 tons of gallium in an aqueous solution of gallium chloride and hydrochloric acid; the detector will be located in the Gran Sasso Laboratory in Italy. Measurements are not expected to begin until 1990. The Soviet experiment will use 60 tons of gallium metal as a detector in a solar-neutrino laboratory constructed underneath a high mountain in the Bak-san Valley. The Soviet experimenters expect to have a detector operating in 1988 or 1989. The scale of both of these experiments is impressive considering the fact that at the time the experimental techniques were developed the total world production of gallium was only 10 tons per year.

The gallium experiments will furnish unique and fundamental information about nuclear processes in the solar interior and about neutrino propagation. No other solar-neutrino experiment that has been funded so far can detect the low-energy neutrinos from the basic pp reaction (reaction 1 of Table 2). The detection threshold for neutrinos by  $^{71}\text{Ge}$  is 0.233 MeV, well below the maximum energy of the pp neutrinos.

Neutrinos from the basic pp reaction are expected<sup>6</sup>, according to the standard solar and electroweak models, to produce about half (54%) of the computed total capture rate. The other main contributors are  $^7\text{Be}$  neutrinos (26%), and  $^8\text{B}$  neutrinos (11%). The dominant uncertainty is caused by the transitions to excited states whose matrix elements must be inferred from (p, n) measurements. If we were to ignore all of the uncertainties associated with the excited state transitions, the remaining total calculated uncertainty would be only 9% of the total capture rate.

The initial chemical extraction is different in the GALLEX and the Soviet experiments, but the final chemical procedures and the methods of low-level counting will be similar for both collaborations. The comparison of the results from the two experiments will be a valuable check on any possible systematic errors. In the GALLEX experiment the radioactive  $^{71}\text{Ge}$  is removed from the aqueous solution of gallium chloride and hydrochloric acid by purging with gas and the  $^{71}\text{Ge}$  is recovered from the gas by absorption in water. This method was developed at Brookhaven National Laboratory and a pilot (1.3 ton Ga) experiment was built and performed<sup>51</sup>. The GALLEX collaboration will use a single vessel to contain the gallium chloride solution. The extracted  $^{71}\text{Ge}$  will be converted to germane ( $\text{GeH}_4$ ) and measured in a miniature gas proportional detector with pulse-height and pulse rise-time analysis. This group will receive their full amount of gallium in late 1989 and expect to begin measurements in 1990. For the Soviet experiment (which has a small number of American collaborators from Los Alamos and the University of Pennsylvania) the germanium extraction process involves mixing the metal with a dilute hydrochloric acid solution in the presence of hydrogen peroxide as an oxidizing agent. The process is carried out in ten mixers each containing 6 tons of gallium metal. The hydrochloric acid containing the extracted  $^{71}\text{Ge}$  is concentrated by boiling; hydrochloric acid is then added until the solution is 8–9 M in HCl. The  $^{71}\text{Ge}$  is removed from this solution by purging with gas collected in water, a procedure similar to the primary  $^{71}\text{Ge}$  separation used in the gallium chloride process. The process used by the Soviet group is slower and more cumbersome to perform, but is also capable of obtaining a high recovery yield (90–95%). Because large volumes of hydrochloric acid are used it is necessary to recycle and concentrate the acid. The technology has been well developed by the Soviet team. The measurement of the  $^{71}\text{Ge}$  radioactivity will also use the proportional counter method with pulse height and pulse rise-time discrimination. Both the GALLEX and Soviet experiments are well supported, and we can look forward to fundamental results from these experiments.

### Next generation experiments

Two powerful new detectors are being developed as next-generation experiments. They are a 3-kt detector of liquid argon<sup>52,53</sup>, to be placed in the Gran Sasso underground laboratory in

northern Italy, and a proposed<sup>54,55</sup> 1-kt detector of heavy water ( $\text{D}_2\text{O}$ ) to be placed in an INCO nickel mine near Sudbury, Ontario (Canada). The argon detector is primarily an Italian experiment with (so far) limited American participation; the deuterium experiment is a collaboration between Canadian, American and English scientists. The Italian funding for the argon experiment has already been approved and the detector could begin taking data around 1990. If funded promptly, the deuterium detector could begin taking data in 1991.

These experiments are sensitive to  $^8\text{B}$  and hep neutrinos, but the other solar neutrinos will be below the energy threshold (expected to be several MeV) set to eliminate unwanted backgrounds. Fortunately,  $^8\text{B}$  and hep neutrinos are produced in different regions of the Sun and are therefore affected differently by some of the most often discussed 'non-standard' solar models. The two sources will provide complementary information about the Sun. The fact that there are two different sources increases the likelihood that a solar neutrino signal of one type or another will be detected. The expected rates vary from several hundred to several thousand events per year in each detector, depending upon the absolute flux of the incident neutrinos and on their flavours<sup>6</sup>.

Both the argon and the deuterium experiments use multi-purpose detectors, which will study solar neutrinos by neutrino capture (sensitive only to the solar  $\nu_e$ , whose flavour is unchanged in transit to the Earth) and by electron-neutrino scattering (cross section for flavour-changed neutrinos is about one seventh the cross section for  $\nu_e$ ). The energy of individual neutrino events will be measured in real time, permitting the determination of the shape of the incident spectrum. The solar origin of the events will be evident from the angular distribution (with respect to the Earth-Sun direction) of the electrons scattered by the neutrinos. It seems likely that the deuterium experiment will be made sensitive to a 'flavour-blind' detection mode in which deuterium nuclei are disintegrated into their constituent neutrons and protons without changing the charge of the nucleons. This so-called 'neutral current mode' is equally sensitive to neutrinos of any type (flavour) and will therefore provide a crucial overall calibration of the flux of solar neutrinos. The ratio of event rates for the charged-current (absorption) process and the neutral-current (disintegration) process could be a sensitive indicator of otherwise inaccessible effects of neutrino transformations.

The interaction cross sections applicable to these experiments can be calculated accurately. Therefore almost all of the total uncertainty in the predicted rates is determined by the uncertainty in the absolute solar production rate of  $^8\text{B}$  and hep neutrinos and by the unknown weak interaction effects on neutrino propagation.

Most recently an experiment has been proposed<sup>56</sup> that would use  $^{11}\text{B}$  as a detector and which could, in principle, observe simultaneously the neutral current, electron scattering and capture (charged-current) reactions. Various detector environments are currently being investigated.

### Detection of the pp neutrinos

Three detectors are being developed to observe the basic (low-energy) pp neutrinos (see the fundamental reaction 1 of Table 2) by electronic methods.

Two of the detectors will operate at very low temperatures and will detect neutrino-electron scattering. A crystalline silicon detector<sup>57</sup> will function as a low-temperature (millikelvin) bolometer measuring the energy deposited by individual neutrinos. A tabletop-sized detector is currently being developed to test the design concepts. A superfluid helium detector has been proposed<sup>58</sup> to study the excitations (primarily rotons) produced in the fluid when neutrinos are scattered by individual electrons. The technical development required to test the validity of the design concepts could be achieved for about \$100,000. A full-scale experiment will be more expensive because it would



require  $\sim 10$  tons of superfluid helium maintained at a temperature  $\sim 20$  mK.

In addition, an  $^{115}\text{In}$  detector is being developed by several European groups<sup>59-61</sup>. This detector will work as a liquid scintillator and will register neutrinos captured by  $^{115}\text{In}$  to produce  $^{115}\text{Sn}$ . The detector is in principle capable of measuring accurately the incident spectrum of pp neutrinos (see Fig. 1), although natural radioactive backgrounds remain a serious problem.

## A bromine radiochemical experiment

A bromine experiment would be a natural successor to the  $^{37}\text{Cl}$  experiment because it could be performed inexpensively using the same tank and experimental location and would involve similar chemical techniques. This experiment uses the low-threshold (0.47 MeV) reaction  $\nu + ^{81}\text{Br} \rightarrow e^- + ^{81}\text{Kr}^* \rightarrow ^{81}\text{Kr}$ , yielding the long-lived  $^{81}\text{Kr}$  with a mean life of  $3.0 \times 10^5$  years. Because the product nucleus is effectively stable on laboratory timescales, a method different from the usual radioactive detection had to be developed. About a decade ago, work was begun that ultimately showed that one could measure the expected few hundred atoms of  $^{81}\text{Kr}$  by resonance ionization spectroscopy<sup>62</sup>. The combined  $^8\text{B}$  and  $^7\text{Be}$  solar neutrino fluxes could be measured with a year's exposure of 800 tonnes of a bromine compound ( $\text{CH}_2\text{Br}_2$ ). The recovery of  $^{81}\text{Kr}$  is easily accomplished by a helium purge after the identical procedures used in the chlorine experiment.

The experiment was originally conceived<sup>63</sup> as primarily sensitive to  $^7\text{Be}$  neutrinos. However, recent work on the absorption cross section to excited states, calibrated by moderate-energy

(p, n) experiments<sup>64</sup>, has shown that transitions to various excited states of the daughter nucleus  $^{81}\text{Kr}$  can greatly increase the effective cross section for the higher-energy  $^8\text{B}$  neutrinos. By the most recent calculations<sup>6</sup>, the  $^8\text{B}$  neutrinos contribute  $\sim 55\%$  of the expected capture rate and  $^7\text{Be}$  neutrinos  $\sim 31\%$ . The relatively large uncertainty in the theoretical predictions ( $\sim 50\%$ ), resulting from inaccurately known neutrino absorption cross sections, limits the inferences that would be possible from a bromine experiment. Of course, if the electron scattering and direct absorption experiments discussed above show that the  $^8\text{B}$  neutrino flux is much below the predictions of the standard solar model, then the theoretical justification for the experiment will be improved. The  $^{81}\text{Br}$  detector would then be primarily sensitive to  $^7\text{Be}$  neutrinos, the only detector whose feasibility is established which could signal out the neutrinos from this important source. Moreover, the cross section uncertainties would be significantly reduced (to  $\sim 30\%$ ). In any event, a  $^{81}\text{Br}$  experiment would provide a valuable constraint on the incident spectrum of solar neutrinos.

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# Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB

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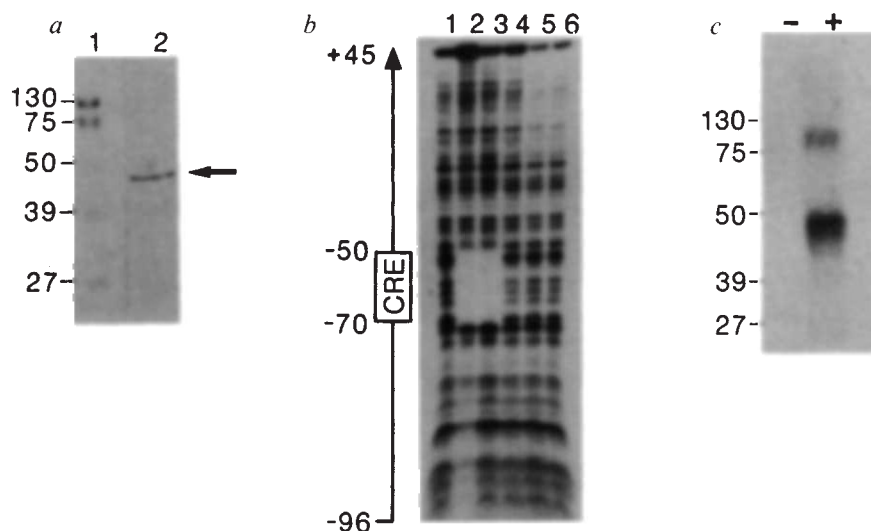
*A nuclear protein, CREB, has been isolated from rat brain and shown to stimulate transcription of the cyclic AMP-responsive gene somatostatin as a dimer. Biochemical analysis suggests that dimerization and transcriptional efficacy of CREB protein in vitro are regulated by phosphorylation. These findings demonstrate that cellular signals can modulate gene expression by regulating the covalent modification of pre-existing nuclear factors.*

CYCLIC AMP (cAMP) mediates the hormonal induction of numerous eukaryotic genes through a conserved cAMP response element (CRE)<sup>1</sup>. We have previously characterized a nuclear phosphoprotein (CREB) with a relative molecular mass ( $M_r$ ) of 43,000 (43K) which binds with high affinity to the CRE of the rat somatostatin gene<sup>2</sup>. Phosphorylation of CREB in response to cAMP activation appears to be necessary for transcriptional induction *in vivo*<sup>1,2</sup>. We now report that purified CREB can stimulate somatostatin gene transcription in nuclear extracts of PC12 cells in an *in vitro* assay. To distinguish between different forms of CREB associated with transcriptional activity, we have used a gel retardation assay. In both crude nuclear

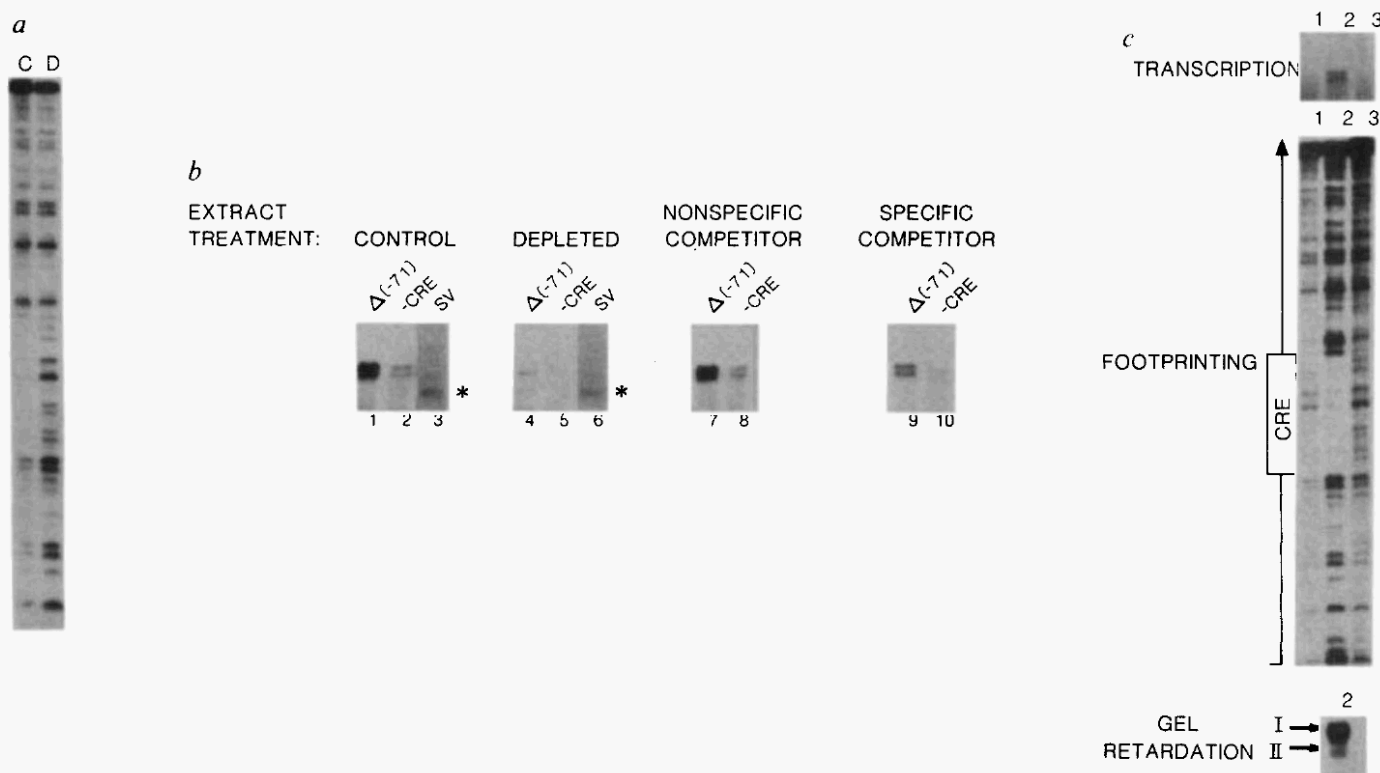
extracts and purified preparations of CREB, we observed what appeared to be monomeric and dimeric forms of the protein-DNA complex. Treating CREB with phosphatase caused a dramatic reduction in the level of dimer activity. In contrast, a significant increase in this dimeric complex was observed after phosphorylating CREB *in vitro* with protein kinase C but not protein kinase A. Fusion genes of somatostatin and chloramphenicol acetyltransferase (CAT) containing mutant CREs which did not recognize the dimer had reduced transcriptional activity *in vitro* and were significantly less responsive to cAMP *in vitro*. Treating *in vitro* transcription extracts with protein kinase A caused a 20-fold increase in CRE-dependent transcrip-

**Fig. 1** Analysis of CREB purified from rat brain. *a*, Silver-stained SDS-polyacrylamide gel of CREB protein after purification. Lane 1, relative-molecular-mass marker; lane 2, CREB protein. *b*, DNase I footprinting analysis of the human *c-fos* CRE following renaturation of CREB activity from an SDS-polyacrylamide gel. Lane 1, no protein; lane 2, purified CREB protein before SDS-PAGE; lanes 3-6, protein fractions eluted from the SDS-gel with approximate relative-molecular-mass of 40-50K, 50-60K, 60-75K, 80-120K, respectively. Position of CRE shown diagrammatically. *c*, Autoradiogram of SDS-polyacrylamide gel showing UV cross-linking of uniformly labelled somatostatin promoter fragment with (-) no protein or (+) purified CREB protein. Ticks alongside gel indicate positions of size markers with  $M_r$ s as indicated.

**Methods.** Nuclei from 500 g rat brains were prepared by homogenizing the tissue with a Tekmar SD-45K homogenizer<sup>8</sup> in two volumes SHE buffer (0.6 M sucrose, 10 mM HEPES pH 7.9, 25 mM KCl, 1 mM EDTA, 10% glycerol with 100 U ml<sup>-1</sup> Trasylol, 1-mM dithiothreitol, 1-mM phenylmethylsulphonyl fluoride). The homogenate was filtered through polypropylene mesh and centrifuged at 14,000g for 25 min. The crude nuclear pellet was washed twice with two volumes SHE buffer and resuspended in two volumes of the same buffer. Nuclear extract was then prepared as described previously for PC12 cells<sup>2</sup>. This extract was applied batchwise to 30 ml DEAE sepharose resin equilibrated in TM buffer<sup>2</sup>. After incubating for 1 h on a rotary wheel, the resin was removed and the flow through fraction, containing CREB activity, was collected. This fraction was then applied to two (10 ml) CRE affinity columns and CREB activity was eluted. DNA affinity columns were constructed as previously described<sup>2</sup>. After dialysis against TM buffer, the CRE-affinity column eluate was applied to a 10-ml AP-1 affinity column. The double stranded AP-1 oligonucleotide contains somatostatin promoter sequences from -60 to -37 except for deletion of a single guanine residue at position -44 which generates a consensus AP-1 site -TGACTA<sup>9</sup>. Because CREB binds the AP-1 sequence with 5-10 fold lower affinity (data not shown), we used an AP-1 DNA affinity column to purify CREB away from contaminants which eluted from the CRE affinity column at high salt. CREB activity from the AP-1 affinity column fractionated with the 0.2M KCl wash fraction. This sample was directly applied to 3 (1 ml) CRE affinity columns and the activity was eluted as before. The eluate was dialysed and examined for purity by SDS-PAGE. Purified CREB (100 footprint units for analytical gel and 1,000 footprint units for renaturation experiments) was precipitated with 10% TCA, washed with acetone, and electrophoresed under reducing conditions on 8% SDS-polyacrylamide gels alongside pre-stained standards (Biorad). For renaturation experiments, gel slices were removed and eluted overnight at 4°C in two volumes elution buffer (50 mM Tris, pH 7.9, 0.1 mM EDTA, 0.1% SDS, 5 mM DTT, 150 mM NaCl). Samples were then precipitated with five volumes of acetone at -20°C to remove SDS, renatured as previously described<sup>10</sup>, and assayed for footprinting activity. Non-coding strand *c-fos* DNA probe for footprinting assays was a 141-base pair (bp) 3'-end-labelled fragment, extending from -96 to +45. The fragment was generated by digesting plasmid pc-fos<sup>H</sup> (gift of P. Herrlich and M. Karin) with restriction enzymes *EcoRI* and *XbaI*. Ultraviolet cross-linking experiments were performed as described previously<sup>2</sup>.







**Fig. 2** Transcriptional efficacy of CREB protein. *a*, CRE-footprinting activity of control (C) or CREB-depleted (D) extracts using 20  $\mu$ g total protein each. *b*, Primer extension products of *in vitro* transcription reactions using somatostatin-CAT fusion gene plasmids  $\Delta(-71)$  CAT<sup>1</sup>, designated  $\Delta(-71)$ , and (-CRE) CAT, designated (-CRE) and SV40-tk plasmid (SV). Arrow points to size of expected products from somatostatin-CAT genes. Asterisks indicate expected position of SV-tk transcripts. Transcriptions were performed in PC12 nuclear extracts. Control: untreated extract (lanes 1–3). Depleted: extract depleted of CREB activity (lanes 4–6). Non-specific competitor: poly(dI·dC) (lanes 7 and 8). Specific competitor: CRE oligonucleotide DNA (lanes 9 and 10). *c*, Binding activity and transcriptional efficacy of CREB following renaturation from SDS-polyacrylamide gel. Lanes 1–3: protein fractions eluted from gel with molecular weights, relative to prestained standards, of 35–40K, 40–45K and 45–50K respectively. Top panel, *in vitro* transcription from plasmid  $\Delta(-71)$  CAT using depleted PC12 nuclear extract supplemented with fractions 1, 2 or 3. Middle panel, footprinting assay of fractions 1, 2 and 3 using the 5'-end-labelled somatostatin promoter fragment. Position of CRE is shown diagrammatically. Lower panel, gel retardation assay of reactions using a 5'-end-labelled CRE oligonucleotide probe. Complexes I and II as indicated.

**Methods.** PC12 nuclear extracts for *in vitro* transcription reactions were prepared as described by Dignam *et al.*<sup>11</sup>. *In vitro* transcriptions and primer extension reactions were performed according to Jones *et al.*<sup>5</sup> and quantitated by scanning densitometry. Primer was a 25-base oligonucleotide containing non-coding somatostatin sequence extending from +31 to +55. PC12 extract was depleted of CREB by passing the extract twice over a 1-ml CRE affinity column. Column fractions were analysed by footprinting assay to verify depletion of CREB. Flow-through fractions depleted of CREB activity were then used directly for *in vitro* transcriptions. For reactions using DNA competitors, 100 ng of either poly(dI·dC) or CRE oligonucleotide, corresponding to a 100-fold molar excess over template DNA, was added to individual samples. (-CRE) CAT plasmid was constructed by digesting  $\Delta(-48)$  CAT plasmid<sup>1</sup> with *Aat*II, removing 3' tails with T4 DNA polymerase, and religating with T4 DNA ligase. Gel renaturation experiments were performed as described in the legend to Fig. 1. Renatured samples were divided into three aliquots and assayed simultaneously for transcription, footprinting and gel retardation activities. Somatostatin DNA probe for footprinting reactions was a 5'-end-labelled 145-bp coding-strand fragment generated by digesting plasmid  $\Delta(-71)$  CAT with restriction enzymes *Hind*III and *Ava*II<sup>2</sup>. Gel retardation assays were adapted from Staudt *et al.*<sup>12</sup>. Nuclear extract (crude and purified) was added to 5'-end-labelled double-stranded CRE oligonucleotide probe (0.4 ng) in buffer A (polyvinylalcohol ( $M_r$  10K), 12.5 mM Tris, pH 7.9, 6 mM MgCl<sub>2</sub>, 5% glycerol, 0.05% Nonidet P40, 50 mM KCl). Under these conditions, DNA probe was in vast excess of CREB protein. After incubating samples at room temperature for 15 min, protein-DNA complexes were resolved on non-denaturing 6% polyacrylamide gels containing 50 mM Tris base, 380 mM glycine, 2 mM EDTA (pH 8.5). Gels were pre-electrophoresed for 10 min at 10 V cm<sup>-1</sup>. Samples were then loaded and run at the same voltage for 3 h. Gels were dried and visualized by autoradiography. Quantitative data for binding curves were obtained by removing and counting individual bands.

tion. Two-dimensional tryptic peptide maps showing phosphorylation of CREB protein at multiple sites suggest dual regulation of CREB dimerization and transcriptional efficacy.

Somatostatin is a brain peptide which, in addition to its role as a hypophysiotropic hormone, functions as a neuromodulator in nerve cells within the cortex and limbic system<sup>3</sup>. Because somatostatin is highly expressed in brain<sup>4</sup>, we examined this tissue as a potentially more abundant and biologically relevant source of CREB protein than PC12 cells. After purifying CREB, we observed a single major band of 43K on SDS-polyacrylamide gels (SDS-PAGE) which migrated with the same relative molecular mass as CREB previously isolated from PC12 cells<sup>2</sup> (Fig. 1a). To identify the purified 43K protein as CREB, we assayed individual SDS-PAGE fractions for footprinting

activity. Only the 43K band possessed *c-fos* CRE (Fig. 1b) (Herrlich *et al.*, manuscript in preparation) and somatostatin CRE binding activity (Fig. 2c). Using this protocol we obtained between 2.5 and 5.0  $\mu$ g of intact CREB protein from brain tissue (500 g).

### Transcriptional efficacy

To determine whether CREB can stimulate gene transcription, we used an *in vitro* transcription assay on nuclear extracts of PC12 cells. The somatostatin-CAT fusion gene,  $\Delta(-71)$  CAT, which contains an intact CRE, directed efficient and accurate RNA polymerase II-dependent transcription (Fig. 2b, lane 1). Removing the CRE, as in the (-CRE) CAT fusion gene, caused a 7-fold reduction in the level of transcription (Fig. 2b, lane 2).

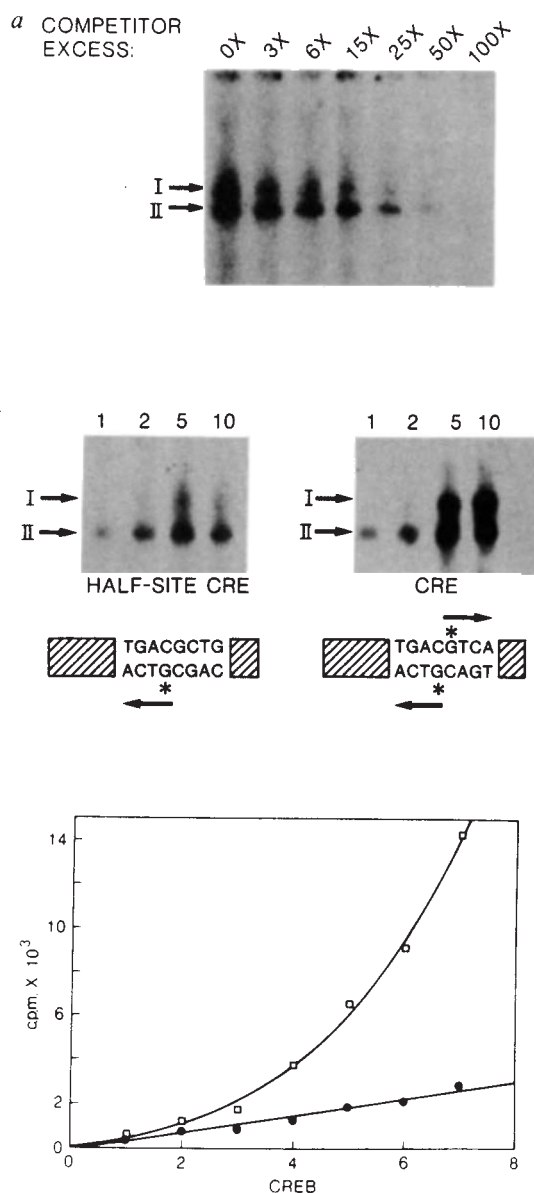
To confirm that transcription from the somatostatin promoter in these nuclear extracts was dependent on CRE, we included either specific (CRE oligonucleotide) or nonspecific poly(dI·dC) competitor DNAs. Transcription from the  $\Delta(-71)$  CAT template was decreased by 10% with poly (dI·dC) competitor (Fig. 2b, lane 7) compared to a 3-fold reduction with CRE competitor (Fig. 2b, lane 9). In contrast, transcription from the (-CRE) CAT plasmid was decreased by 10% using either competitor DNA (Fig. 2b, lanes 8, 10). Nuclear extracts of PC12 cells which had been depleted of CREB by CRE-affinity column chromatography showed no CRE-footprinting activity (Fig. 2a) and an 8-fold reduction in  $\Delta(-71)$  CAT-directed transcription (Fig. 2b, lane 4). Transcription from the (-CRE) CAT plasmid was decreased by 40% only (Fig. 2b, lane 5). Transcription from a control vector, SV-tk (ref. 5), which contains the SV40 promoter fused to the herpes virus thymidine kinase gene, was similarly unaffected by depletion (Fig. 2b, lanes 3, 6). After adding gel-purified CREB protein to depleted extracts, we observed that the 43K band both stimulated  $\Delta(-71)$  CAT transcription and recognized the somatostatin CRE in footprinting and gel retardation assays (Fig. 2c). Purified CREB was unable, however, to activate (-CRE)CAT-directed transcription when added to depleted extracts (data not shown).

### Dimer binding

A gel retardation assay was used to detect protein-DNA complexes in crude nuclear extracts of PC12 cells and brain tissue (Fig. 3a). After incubating a  $^{32}$ P-labelled CRE oligonucleotide with these extracts and resolving the products on non-denaturing polyacrylamide gels, we observed two complexes of high relative molecular mass. Although both complexes could be specifically competed out by an unlabelled CRE oligonucleotide, the complex of higher relative molecular mass (complex I) had ~10-fold higher affinity than the complex with lower relative molecular mass (complex II). Moreover, the equilibrium binding curve of purified CREB protein for complex II was linear, but exponential for complex I (Fig. 3b, c). These results indicated that complex II represents the monomer form of CREB, whereas complex I represents a multimer. To rule out the possibility that one of these complexes represented either a contaminant or a degraded form of CREB, we assayed CREB activity after SDS-gel purification and observed that the single 43K band could indeed give rise to both complexes (Fig. 2b). At lower concentrations of gel-purified CREB, only complex II was observed (data not shown).

The palindromic nature of the CRE prompted us to ask whether CREB activates transcription by binding to DNA as a dimer. Using methylation interference assays, we observed that CREB binds to the CRE palindrome in a symmetrical way by forming close contacts with a short motif, CGTCA, on each strand of the DNA (data not shown; Fig. 3b). If both CGTCA motifs within the CRE were critical to the formation of complex I, representing a putative CREB dimer, then a half-site CRE oligonucleotide with only one copy of the motif should be incapable of forming this complex. As predicted, the half-site CRE oligonucleotide formed only complex II, the monomer (Fig. 3b). To confirm that complex I contains a dimer, and complex II a monomer, we performed an ultraviolet cross-linking experiment<sup>2</sup> with purified CREB protein. We observed two specific protein-DNA adducts of ~45K and 90K which were specifically competed out by unlabelled CRE oligonucleotide (Fig. 1c and data not shown). The relative molecular masses of these complexes are consistent with monomer and dimer forms of CREB, respectively.

To determine whether complexes I and II represent DNA-bound forms of CREB with different transcriptional activities *in vivo*, we transfected PC12 cells with somatostatin-CAT fusion genes containing 0, 1 or 2 CGTCA motifs. CAT activity derived from the wild-type template  $\Delta(-71)$  CAT, containing two motifs, was induced 14-fold by forskolin, a direct activator of adenylyl



**Fig. 3** Analysis of CREB protein by gel retardation assay. **a**, Autoradiogram showing assay of crude PC12 nuclear extract using 5'-end-labelled CRE oligonucleotide probe. Fold-excess of unlabelled CRE competitor DNA shown above each lane. Complexes I and II as indicated. **b**, Gel retardation assay using purified CREB. Numbers above each lane indicate amount of CREB added in  $\mu$ l. CRE and half-site CRE, double-stranded oligonucleotides used as DNA probes. Shaded boxes indicate somatostatin promoter sequences from -60 to -48 on 5' end and -42 to -37 on 3' end for each. G residues that make close contact with CREB, as determined by methylation interference assay, are indicated by G\* on sense and anti-sense strands, respectively. Arrows indicate CGTCA motif. **c**, Equilibrium binding curves for complexes I and II.  $^{32}$ P counts in each complex on ordinate plotted against amount CREB protein added (in  $\mu$ l) on abscissa. Open boxes denote c.p.m. from complex I. Closed circles denote complex II. Curves are representative of four duplicate experiments.

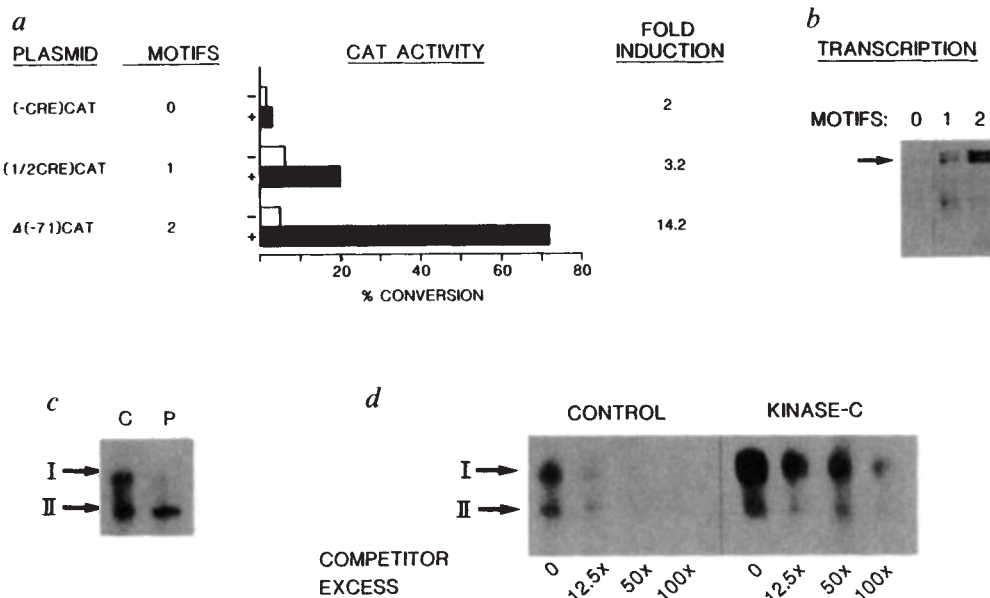
**Methods.** Gel retardation assays were performed as described in the legend to Fig. 2. For methylation interference assays, sense and anti-sense strands of the CRE oligonucleotide were labelled individually, annealed to their unlabelled complementary strands, and then methylated with dimethyl sulphate<sup>13</sup>. Methylated DNA probes were incubated with CREB protein as described above. After electrophoresis, individual complexes were excised and eluted. Samples were then treated with piperidine, denatured and loaded on to 20% urea polyacrylamide gels.



**Fig. 4** Regulation of CREB transcriptional efficacy and binding activity. *a*, Cyclic AMP responsiveness of somatostatin-CAT fusion genes. Individual plasmids with number of CGTCA motifs shown alongside. CAT activity derived from each construct after transfection into PC12 cells shown on bar graph. (–) and (+) denote control or forskolin treatment (10  $\mu$ M). Fold-induction of CAT activity in response to forskolin shown on right.

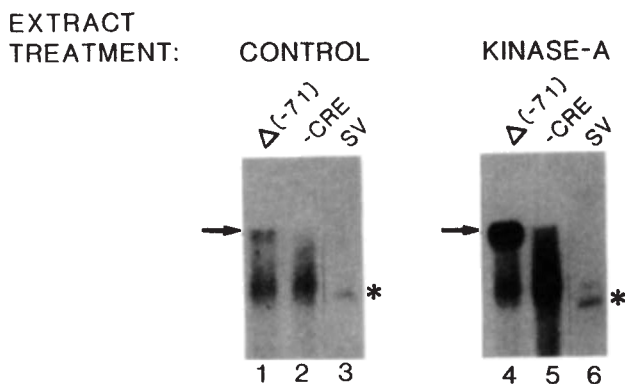
*b*, *In vitro* transcription from somatostatin-CAT gene plasmids containing 0 ((–CRE)CAT), 1 ((1/2 CRE)CAT) or 2 ((CRE)CAT)-CGTCA motifs. *c*, Effect of phosphorylation state on complex formation. Gel retardation assay of purified CREB following phosphatase treatment. C, control; P, CREB treated with calf alkaline phosphatase. *d*, CREB binding activity: untreated protein (control); treated with protein kinase C (kinase-C). Fold-excess of unlabelled CRE competitor shown below each lane. Complexes I and II, as indicated.

**Methods.** (1/2 CRE) CAT (1 motif) and (CRE) CAT (2 motifs) plasmids were constructed by inserting 1/2 site CRE and CRE oligonucleotides (Fig. 3b) into *Aat*II cut, T4 DNA polymerase digested  $\Delta(-48)$  CAT<sup>1</sup>. Transfection experiments and CAT assays were performed and quantitated as previously described<sup>1</sup>. Purified CREB protein was treated with 20 U calf intestinal alkaline phosphatase (Boehringer Mannheim) in Buffer A (see Fig. 3) at room temperature for 10 min. Klenow-labelled CRE oligonucleotide probe was then added and gel retardation assays were performed as in Fig. 3. For protein kinase C reactions<sup>14,15</sup>, 2.4 ng CREB protein was incubated with purified protein kinase C or no enzyme (control) for 7 min at 30 °C in 25 mM HEPES, pH 7.5, 10 mM MgCl<sub>2</sub>, 60 ng ml<sup>–1</sup> phosphatidylserine, 2.8  $\mu$ g ml<sup>–1</sup> diacylglycerol, 0.5 mM CaCl<sub>2</sub>, 25  $\mu$ M ATP. For gel retardation assays, CRE probe was added to control (no enzyme) or protein kinase C-treated samples, and binding reactions were performed as in Fig. 3.



**Fig. 5** Effect of protein kinase A on transcription *in vitro*. Primer extension products using somatostatin-CAT fusion gene plasmids  $\Delta(-71)$  CAT, designated  $\Delta(-71)$  (lanes 1, 4), and (–CRE) CAT, –CRE (lanes 2, 5). SV, SV-tk plasmid containing SV-40 promoter fused to herpesvirus thymidine kinase structural gene (lanes 3, 6). Control, untreated extract. Protein kinase A, extract treated with purified catalytic subunit of cAMP-dependent protein kinase A. Arrows point to size of expected products from somatostatin-CAT genes. Asterisks indicate expected position of SV-tk transcripts.

**Methods.** *In vitro* transcription reactions were performed as described in Fig. 2. For protein kinase A treatment, 1  $\mu$ g purified catalytic subunit was added directly to transcription extracts without additional modifications.



cyclase (Fig. 4a). In contrast (–CRE) CAT (no motifs), and (1/2 CRE) CAT (one motif) fusion genes were induced only 2- and 3-fold, respectively. Likewise, *in vitro* transcription directed by the mutant plasmids, (–CRE) CAT and (1/2 CRE) CAT, was significantly reduced compared to the wild-type template (Fig. 4b). The inability of these mutant CREs to form complex I in gel retardation assays (Fig. 3b) is therefore correlated with diminished transcriptional activity *in vitro* and loss of cAMP responsiveness *in vivo*.

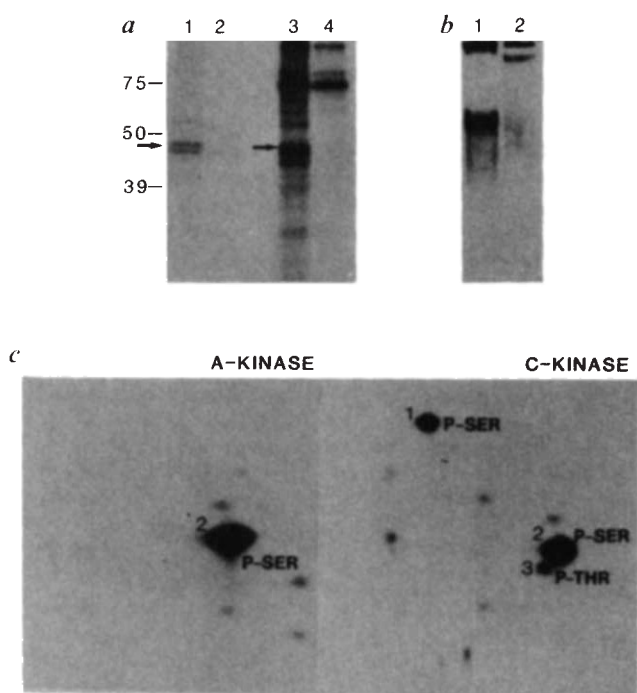
## Phosphorylation

Previous experiments showing that CREB is a phosphoprotein<sup>1,2</sup> prompted us to ask whether complex formation was dependent on the phosphorylation state of CREB. Treating affinity-purified CREB with alkaline phosphatase caused a substantial decrease in levels of complex I but had no effect on complex II (Fig. 4c). This finding suggested that phosphorylation of CREB by an appropriate kinase should conversely promote the formation of complex I. Because CREB is efficiently phosphorylated *in*

*vitro* by both protein kinase A and protein kinase C (Fig. 6a), we tested CREB activity following treatment with these enzymes. Although CREB binding activity was unchanged following treatment with protein kinase A (data not shown) protein kinase C caused a marked induction of complex I, the putative dimer (Fig. 4d).

To determine the effects of protein kinase A on the transcriptional efficacy of the CREB protein, we treated PC12 extracts with this enzyme and performed *in vitro* transcription assays. Protein kinase A treatment induced CRE-dependent transcription by ~20-fold (Fig. 5, lanes 1, 4) but had no effect on (–CRE) CAT expression. SV-tk expression was increased only 2-fold (Fig. 5, lanes 2, 3, 5, 6). These results indicate that transcriptional induction by protein kinase A is confined to promoters containing a CRE. We were unable to test the effects of kinase C because essential components of the kinase buffer, most notably phosphatidylserine, interfered strongly with transcription.

Previous experiments showing that CREB is phosphorylated in PC12 cells prompted us to analyse the sites on the protein



**Fig. 6** Phosphorylation and two dimensional tryptic peptide mapping of  $^{32}\text{P}$ -labelled CREB protein. *a*, Autoradiogram of purified CREB labelled with [ $\gamma$ - $^{32}\text{P}$ ]ATP after treatment with protein kinase A (lanes 1, 2) or protein kinase C (lanes 3, 4). Lane 1, CREB plus protein kinase A. Lane 2, protein kinase-A alone. Lane 3, kinase-C alone. Arrows point to labelled CREB protein. *b*, Gel retardation assay of  $^{32}\text{P}$ -labelled CREB after elution and renaturation from SDS-PAGE. Lane 1, CREB plus unlabelled CRE oligonucleotide; lane 2, CREB alone. *c*, Two-dimensional tryptic peptide maps of  $^{32}\text{P}$ -labelled CREB. Left panel, CREB phosphorylated by protein kinase A. Right panel, CREB phosphorylated by kinase-C. Tryptic phosphopeptides 1, 2 and 3 referred to in text. P-SER, P-THR, phosphoserine and phosphothreonine residues determined by phosphoamino-acid analysis.

**Methods.** CREB was treated with protein kinase A as described previously<sup>2</sup>. Protein kinase C reactions were performed as described in Fig. 4. Gel retardation assays were as outlined in Fig. 2, except that 0.4 ng unlabelled CRE oligonucleotide was used. Two-dimensional tryptic peptide mapping and phosphoamino-acid analysis were performed according to Gould *et al.*<sup>16</sup>.

that are phosphorylated *in vitro* by protein kinase A and protein kinase C. CREB purified from brain tissue was phosphorylated by both enzymes (Fig. 6*a*). To demonstrate that the 43K  $^{32}\text{P}$ -labelled band was in fact CREB, we performed gel renaturation experiments and showed, by a gel retardation assay with unlabelled CRE oligonucleotide, that the  $^{32}\text{P}$ -labelled renatured protein had CRE binding activity (Fig. 6*b*, lane 1). No such complex was observed in the absence of CRE oligonucleotide (Fig. 6*b*, lane 2). Two-dimensional tryptic peptide mapping of  $^{32}\text{P}$ -labelled CREB showed that protein kinase A stimulates phosphorylation of a single peptide, referred to as peptide 2 (Fig. 6*c*, left panel). Protein kinase C treatment not only induced phosphorylation of peptide 2, but additionally stimulated phos-

phorylation of peptides 1 and 3 (Fig. 6*b*, right panel). Phosphoamino-acid analysis of peptides 1 and 2 revealed phosphoserine, whereas peptide 3 contained phosphothreonine.

## Discussion

Cyclic AMP-inducible transcription in PC12 cells is dependent on protein kinase A activation<sup>1</sup>. We were surprised to find, therefore, that protein kinase C, but not protein kinase A, stimulated the formation of CREB dimer. Cambier *et al.*<sup>6</sup> have recently observed that cAMP stimulates the translocation of protein kinase C to the nuclei of B lymphocytes. This translocation appeared to be specific for cAMP because increased phosphoinositol (PI) turnover had no effect on nuclear levels of protein kinase C. Although our results do not show whether cAMP responsiveness is mediated by protein kinase C translocation in PC12 cells, it will be interesting to determine whether nuclear translocation of protein kinase C also occurs in these cells and, if so, whether translocation is inhibited in mutant lines which are deficient in protein kinase A.

The ability of protein kinase A to stimulate CRE-dependent transcription without influencing dimerization suggests dual regulation of CREB binding activity and transcriptional efficacy. Two-dimensional tryptic peptide maps demonstrating that protein kinase C phosphorylates CREB at different sites from protein kinase A support this model. Thus phosphorylation of peptides 1 and 3 is correlated with increased dimerization of CREB.

In prokaryotes, numerous transcription factors bind to palindromic sequences as dimers, the monomers having greatly reduced DNA-binding affinity and transcriptional efficacy. Similarly, our results suggest that CREB exists in equilibrium between a pool of transcriptionally active dimers (complex I) and relatively inactive monomers (complex II). Phosphorylation of CREB shifts this equilibrium towards dimer formation. We do not yet know whether CREB dimers form spontaneously or only after binding to DNA. The exponential binding curve for complex I suggests that very small changes in monomer concentration or in monomer-monomer binding affinity can cause large changes in the level of CREB dimer, and consequently in transcriptional efficacy. Conversely, inactivation by phosphatases would shift the equilibrium binding conditions towards monomer formation and reduced transcriptional activity.

Recent findings by Lee *et al.*<sup>7</sup> suggest that CREB may also mediate the *trans*-activation of adenovirus type-2 early promoters by E1A. If so, E1A protein may also activate CREB dimerization, either by increasing the local concentration of monomers, or by stimulating CREB phosphorylation.

Our results provide a testable model with which to investigate further the mechanism of transcriptional induction by cAMP. The isolation of cDNA clones encoding CREB will help to elucidate the mechanism by which this protein stimulates transcription.

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# Regulation of calcium influx by second messengers in rat mast cells

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*Biphasic increases in the free intracellular calcium concentration, consisting of a large initial transient followed by a sustained elevation, are frequently observed in non-excitabile cells following stimulation. In rat peritoneal mast cells a cAMP- and Ca-activated chloride current can interact with IP<sub>3</sub>-dependent calcium influx to provide the sustained elevation of intracellular Ca concentration following transient IP<sub>3</sub>-induced release of calcium from intracellular stores. This novel combination of second messenger systems provides a flexible means to modulate calcium-dependent processes such as exocytosis.*

CHANGES in intracellular calcium concentration are important in the regulation of a variety of cellular functions, including secretion, contraction and hormone actions. Increases in  $[Ca]_i$  in excitable cells arise mainly via voltage-activated calcium channels, whereas in non-excitabile cells release of intracellular calcium by the second messenger inositol trisphosphate (IP<sub>3</sub>) is prominent<sup>1</sup>. In addition to this typically transient increase in  $[Ca]_i$ , which is independent of extracellular calcium, many non-excitabile cells show a sustained phase of elevated  $[Ca]_i$  due to influx of extracellular calcium<sup>2,3</sup>. Electrophysiological and pharmacological evidence suggests that this influx is mediated by second messengers rather than by classic voltage-dependent calcium channels. A potential role in mediating calcium influx has been attributed to  $[Ca]_i$  (ref. 4) and IP<sub>3</sub> (refs 5–7). Also IP<sub>4</sub> may be involved in calcium influx<sup>8,9</sup>, and cAMP can influence calcium influx in non-excitabile cells by unknown mechanisms<sup>10–12</sup>.

We have performed simultaneous patch-clamp and calcium-indicator dye (fura-2) measurements in rat peritoneal mast cells in order to investigate molecular mechanisms underlying second messenger mediated calcium signals. In these non-excitabile cells, previously thought to be devoid of physiologically relevant ion channels<sup>13</sup>, we have discovered three ionic mechanisms that are activated following receptor stimulation and that may enhance secretion by maintaining elevated  $[Ca]_i$ : (1), a voltage-independent cation channel of ~50 pS conductance through which divalents can permeate according to their thermodynamic driving force; (2), a hyperpolarization-driven calcium influx activated both by external stimuli and by intracellularly applied IP<sub>3</sub>—if this influx is mediated by ion channels, they must be very calcium-specific because large changes in  $[Ca]_i$  occur with whole cell currents of 1–2 pA or less; (3) a chloride current of 0.5–1 pS single-channel conductance, activated following external stimulation and also induced by internally applied cAMP and by high  $[Ca]_i$ . This current will clamp the membrane potential of an intact cell to negative values, thus providing driving force for calcium influx through mechanisms (1) and (2).

## Conductance and $[Ca]_i$ following agonist

An overview of the effects of secretory stimulation on  $[Ca]_i$  and on membrane conductance is shown in Fig. 1a. Following stimulation of mast cells by secretagogues such as compound 48/80 or substance P, we observed a large, transient increase in  $[Ca]_i$  that was independent of external calcium. It was often followed by a plateau of elevated  $[Ca]_i$  (0.3–0.8  $\mu$ M) lasting tens of seconds. This latter sustained phase of elevated  $[Ca]_i$  was abolished as external calcium was removed from the bath solution (not illustrated, but see Fig. 4b), suggesting that it results from

influx of extracellular calcium. Changes in voltage had no influence on  $[Ca]_i$  before stimulation, but during the plateau,  $[Ca]_i$  increased with hyperpolarization and decreased with depolarization, further indicating that membrane calcium conductance was increased. As will be pointed out below, the pathway for calcium influx is opened by second messengers rather than being voltage-gated. However, the amount of calcium flowing through this pathway may be termed 'potential-dependent' in that the influx depends on the electrical driving force as long as the pathway remains activated. It is therefore not surprising that membrane hyperpolarization promotes calcium influx, whereas less calcium enters upon depolarization, contrary to the case of excitable cells, where depolarization opens voltage-activated calcium channels. The phenomenon that calcium influx following agonist stimulation is reduced by depolarization and increased by hyperpolarization has been recognized in various non-excitabile cells<sup>3,14–17</sup>.

The most prominent conductance change following stimulation was a slowly developing, outwardly rectifying current that was responsible for the large increase in outward current during positive voltage pulses in Fig. 1a. As will be shown below, this current was carried mainly by chloride ions and will be referred to as the delayed chloride current. Inward currents that might account for the plateau phase of elevated  $[Ca]_i$  were much smaller in amplitude and barely visible in Fig. 1a. However, we did find that secretagogue stimulation increased the activity of 50-pS cation channels through which calcium may permeate (see below), but as discussed later, these channels are likely not the primary source of hyperpolarization-driven calcium influx during the plateau phase. In a few favourable experiments, close inspection of current records revealed small, smooth changes in the DC level on which the above-mentioned channels superimposed. This small inward current may be more closely related to the calcium influx than the large channels. A more detailed description of these currents will follow below.

## cAMP and IP<sub>3</sub> mimic agonist-induced responses

We attempted to 'reconstitute' the physiological responses in the absence of agonist-mediated stimulation by introducing second messengers known to play a role in signal transduction in mast cells, notably IP<sub>3</sub> and cAMP. The effects of dialysing a cell with a pipette solution containing 50  $\mu$ M cAMP are illustrated in Fig. 1b. Cyclic AMP induced a slowly developing current indistinguishable from the delayed chloride current seen after secretagogue stimulation, suggesting that the delayed chloride current in stimulated cells is induced by increased levels of cAMP. This current is, however, likely to be subject to complex regulation, including modulation by  $[Ca]_i$ . Cyclic AMP did not cause changes in  $[Ca]_i$ .

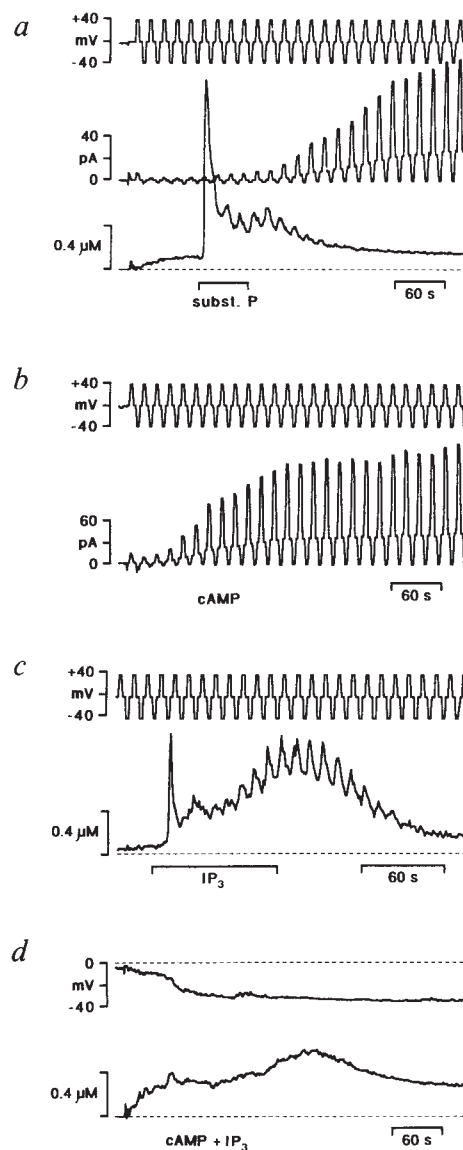
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**Fig. 1** Changes in current and  $[Ca]_i$  in response to agonist stimulation are mimicked by cAMP and  $IP_3$ . *a*, Substance P ( $50 \mu g\ ml^{-1}$ ) was applied for the time indicated. The cell was voltage-clamped to different potentials (top trace) and the resulting currents (middle trace) and changes in  $[Ca]_i$  (bottom trace) are shown. *b*, The cell was perfused with standard internal solution supplemented with  $50 \mu M$  cAMP and subjected to the same voltage protocol. *c*, The cell was initially perfused with standard internal solution. For the time indicated, the cell was dialysed with a solution that contained  $IP_3$  ( $10 \mu M$ ) using micro-pipette perfusion. *d*, The cell membrane potential was monitored in current clamp while cAMP ( $50 \mu M$ ) and  $IP_3$  ( $0.5 \mu M$ ) were included in the pipette-filling solution.

**Methods.** Rat peritoneal mast cells were purified on a percoll gradient as described<sup>38</sup> and suspended in culture medium M199 supplemented with fetal calf serum (10%),  $NaHCO_3$  (45 mM), glucose (2.5 mM), streptomycin ( $0.12\ mg\ ml^{-1}$ ), penicillin ( $0.64\ mg\ ml^{-1}$ ), pH 7.2. Cells were plated onto cover slips placed inside 35-mm culture dishes and stored in an incubator at  $37^\circ C$  and 10%  $CO_2$  for 1–6 h until use. Experiments were performed at  $23$ – $26^\circ C$  in a Mg-rich saline of the following composition (in mM): NaCl 140, KCl 2.5,  $CaCl_2$  2,  $MgCl_2$  5, glucose 11, HEPES-NaOH 10, pH 7.2. In some experiments  $10 \mu M$  DIDS (4,4'-diisothiocyano-2,2'-stilbenedisulphonate) was added to external solutions. Patch-clamp measurements were performed with Sylgard-coated pipettes in the tight-seal whole-cell configuration<sup>39</sup>. Pipette resistance, after filling with the solution given below, ranged between  $3\ M\Omega$  and  $5\ M\Omega$ . The solution for filling pipettes (intracellular solution) contained (in mM): K-glutamate 135, NaCl 20,  $MgCl_2$  1, HEPES-NaOH 10,  $Na_2-ATP$  0.2, GTP 0.3, fura-2 pentapotassium salt 0.1 (Molecular Probes). Occasionally DIDS ( $10 \mu M$ ) was also included. Secretagogues 48/80 ( $5 \mu g\ ml^{-1}$ ) and substance P ( $50 \mu g\ ml^{-1}$ ) were dissolved in extracellular solution and applied directly onto the cell under investigation via pressure ejection from a second pipette. GTP- $\gamma$ -S (kindly provided by Dr F. Eckstein, Göttingen) was used at  $40$ – $100 \mu M$ . Inositol 1,4,5-trisphosphate ( $IP_3$ ), inositol 1,3,4,5-tetrakisphosphate ( $IP_4$ ) (both Amersham, UK) and cAMP (Sigma) were used at the concentrations indicated. The concentration of intracellular calcium  $[Ca]_i$  was measured by use of the fluorescent indicator dye fura-2. Cells were loaded with fura-2 pentapotassium salt by diffusion from the recording pipette. Fluorescence of fura-2 was excited alternately by light at 360 and 390 nm by means of a rotating filter wheel fitted to a slot in the excitation pathway.  $[Ca]_i$  was calculated from the fluorescence ratio<sup>40</sup>. The calibration procedure and details of the measurement are described elsewhere<sup>18,41</sup>. A total of four signals (two fluorescence intensities; DC current and DC voltage) were fed to the computer and sampled every 0.5 s. In addition, signals were recorded on magnetic tape for later noise analysis and single-channel measurements. Single-channel conductance was calculated from variance and mean current at 2–500 Hz bandwidth assuming low opening probability of channels.

When  $IP_3$  was perfused into the cell (Fig. 1c), there were one or more pulsatile increases in  $[Ca]_i$  that were not synchronized with membrane hyperpolarizations. These likely represent  $IP_3$ -induced release of calcium from internal stores<sup>18</sup>. After a delay, transient increases in  $[Ca]_i$  occurred during hyperpolarizing voltage pulses, in a manner similar to the plateau phase of  $[Ca]_i$  following secretagogue stimulation (Fig. 1a). These potential-dependent changes in  $[Ca]_i$  induced by  $IP_3$  required extracellular calcium (see Fig. 4b).

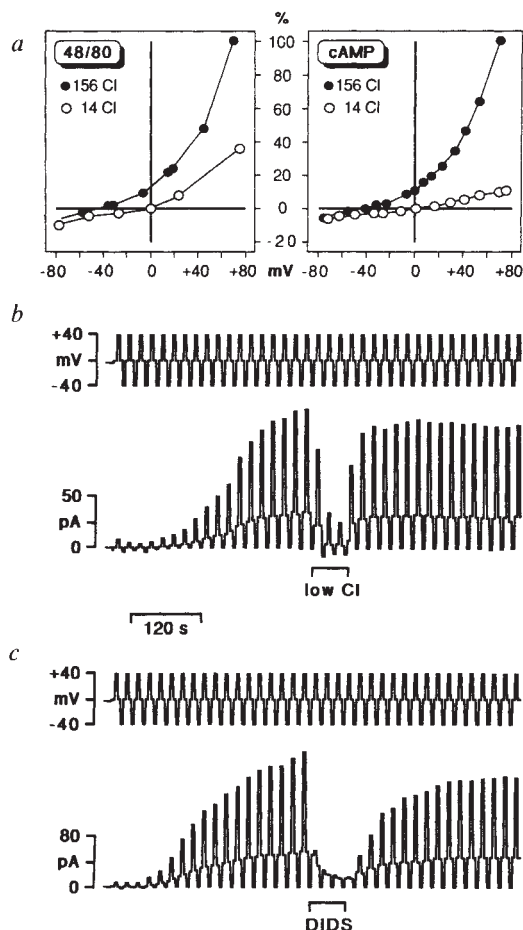
Thus, cAMP mimicked the stimulation-induced delayed chloride current, while  $IP_3$  reproduced the changes in  $[Ca]_i$  that follow stimulation. If cAMP and  $IP_3$  indeed cause the changes in membrane properties that occur with secretagogue stimulation, a combination of cAMP and  $IP_3$  in the pipette solution should mimic the physiological events. The experiment shown in Fig. 1d was carried out to test this under current clamp, with membrane potential allowed to vary according to the currents flowing. The membrane potential following patch rupture was close to 0 mV and slowly hyperpolarized to  $-40$  mV as cAMP diffused into the cell and activated the delayed chloride current, which reverses at about  $-40$  mV (see next section). At this voltage, the driving force for extracellular calcium was substantial, and after a delay,  $[Ca]_i$  increased to produce a plateau



reminiscent of the sustained phase of  $[Ca]_i$  following agonist stimulation. It therefore appears likely that the two second messengers act in concert to bring about the changes in  $[Ca]_i$ :  $IP_3$  opens a pathway through which calcium enters the cell and cAMP activates a chloride current, which provides the driving force for calcium entry.

In experiments where second messengers were perfused into the cell we could regularly obtain consistent and reproducible activation of the various mechanisms. Thus, we could induce calcium influx by  $5$ – $10 \mu M$   $IP_3$  in 79% of the cells ( $n = 68$ ) and the delayed chloride current was activated by cAMP in 77% of the cells investigated ( $n = 96$ ). Responses following agonist stimulation showed some degree of variability across preparations, although being quite uniform among cells of a given preparation. Activation of the cation channels to variable extents was evident in 83% of the cells ( $n = 111$ ). The hyperpolarization-driven plateau phase of elevated  $[Ca]_i$  was seen in 26% of the cells ( $n = 93$ ), the others showing only the initial calcium transient. This variable occurrence of the plateau phase may reflect true variability in the physiological state of mast cells that is meaningful in determining the responsiveness towards secretagogue stimulation. Alternatively, there may be inadvertent variations in our experimental conditions (for example,





**Fig. 2** The properties of the delayed chloride current activated by agonist stimulation and cAMP. *a*, Current-voltage relationships of the delayed chloride current activated by compound 48/80 ( $5 \mu\text{g ml}^{-1}$ ) and cAMP ( $50 \mu\text{M}$ ) with standard external saline (●) and low-chloride saline (○), where NaCl was replaced by Na-glutamate. Current amplitudes were normalized by setting the current amplitude at  $+70 \text{ mV}$  in normal chloride to 100% in each graph (70 pA, 48/80; 110 pA, cAMP). *b*, The delayed chloride current was maximally activated by internal cAMP ( $100 \mu\text{M}$ ). At the time indicated, the cell was flushed with low-chloride external saline as in (*a*). *c*, Same as (*b*), except that the cell was flushed with standard external saline containing  $10 \mu\text{M}$  DIDS. Following removal of DIDS, the current recovered, which contrasts with the irreversible action of DIDS on other chloride channels<sup>19</sup>.

washout) that prevent the manifestation of calcium influx in some cells.

### Delayed chloride current activated by cAMP

A typical current-voltage relationship for the delayed chloride current induced by compound 48/80 is illustrated in Fig. 2*a* (left panel); it shows outward rectification and reversal near  $-40 \text{ mV}$ . As shown in Figs 2*b* and *c*, this current could also be maximally activated by  $50\text{--}100 \mu\text{M}$  internal cAMP in the absence of secretagogue. The current-voltage relation for the cAMP current is shown in Fig. 2*a* (right panel). At  $+50 \text{ mV}$ , the chord conductance of the single channels underlying the delayed chloride current was  $0.5\text{--}1 \text{ pS}$  as determined by noise analysis. When external chloride concentration was reduced from 156 to 14 mM (internal concentration was 11 mM), there was a strong reduction in outward current, and the reversal potential shifted to near  $0 \text{ mV}$  for both agonist-induced and cAMP-induced currents (Fig. 2*a*). The effects of reducing external chloride on the cAMP-induced current are illustrated in Fig. 2*b*. Voltage pulses to three different potentials elicited either no current (at the usual reversal potential of  $-40 \text{ mV}$ ) or large

outward currents under conditions of high external chloride concentration. For the time indicated by the bar, low chloride solution was applied to the cell via local perfusion, shifting the reversal potential to about  $0 \text{ mV}$  and reducing outward current.

Adding 4,4'-diisothiocyano-2,2'-stilbenedisulphonate (DIDS), which has been shown to block chloride channels<sup>19</sup>, strongly reduced the delayed chloride conductance, as demonstrated in Fig. 2*c*. This experiment was carried out under identical conditions as the one in Fig. 2*b*, except that the cell was superfused with an external solution containing  $10 \mu\text{M}$  DIDS, instead of low-chloride solution.

While cAMP was the most potent and reliable activator, the delayed chloride current also frequently developed in non-stimulated cells under a variety of experimental conditions, although it was small and greatly delayed. For example, high  $[\text{Ca}]_i$ , in the range  $0.6\text{--}2 \mu\text{M}$ , was capable of inducing the current (not illustrated), but the onset lagged markedly behind the rise in  $[\text{Ca}]_i$  and maximal activation was obtained only after hundreds of seconds. Elevated  $[\text{Ca}]_i$  may account for the finding that the delayed current also frequently developed in the presence of  $\text{IP}_3$ , as the  $\text{IP}_3$ -induced delayed current was almost completely abolished when internal calcium was buffered to low levels with 2 mM EGTA. Cyclic AMP could fully activate the delayed chloride current even in the presence of 2 mM EGTA (not illustrated), showing that increased  $[\text{Ca}]_i$  is not necessary for the linkage between cAMP and the chloride conductance.

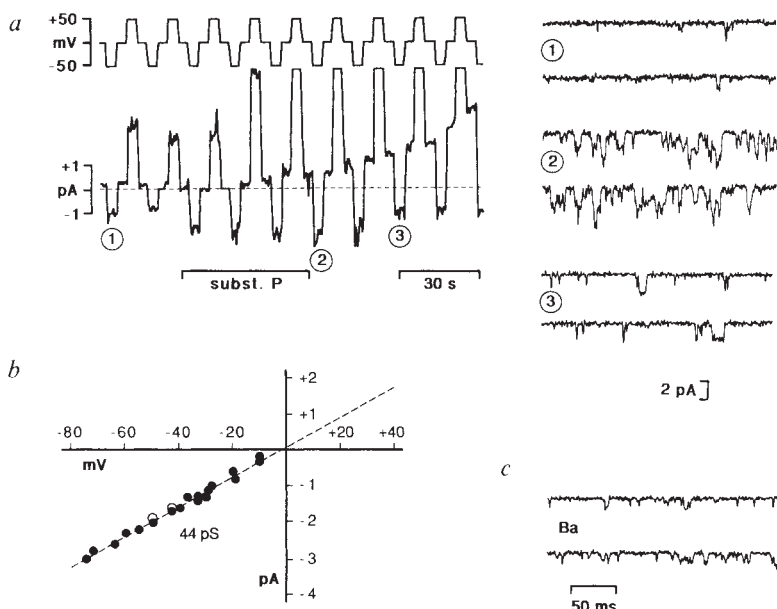
Antigenic stimulation, which is known to cause a transient increase in cAMP levels<sup>20</sup>, induces Ca influx<sup>12</sup>. In the case of 48/80 stimulation, however, cAMP levels either decrease or remain unchanged<sup>21</sup>. Hence, cAMP may play an important role in supporting Ca influx following antigenic stimulation, whereas it may not do so following stimulation with 48/80. It thus appears that another mechanism is responsible for the activation of the delayed chloride current in 48/80-stimulated cells. One may argue that the initial calcium transient induced by  $\text{IP}_3$  activates this current. However, the comparatively rapid onset of the chloride current following stimulation is in contrast to the delayed onset of the current when activated by high  $[\text{Ca}]_i$  alone. This delayed activation by high  $[\text{Ca}]_i$  indicates that the current may not be directly gated by calcium, but possibly by some, at present unknown, calcium-dependent process. In addition, it seems unlikely that cAMP directly activates the chloride current, since cAMP levels in the cytosol (imposed by the intra-pipette solution) are high long before full activation of the delayed current. This delayed gating may reflect the activation of a process secondary to the increase in cAMP, possibly involving phosphorylation processes mediated by cAMP-dependent protein kinase. It is also conceivable that activation of protein kinase C may enhance the delayed chloride current following agonist stimulation. Chloride channels activated by calcium and cAMP have been described in epithelial cells<sup>22</sup>, but their single-channel conductance is larger<sup>23</sup> than that of the channels underlying the delayed chloride current.

The delayed chloride current, which was present in the vast majority of cells, was preceded in about 10% of the cells by another, fast chloride current that was activated directly by  $[\text{Ca}]_i$  and was particularly prominent during the initial calcium transient following secretagogue stimulation. This chloride current was clearly distinct from the delayed current, since it activated and deactivated extremely fast, closely following the changes in  $[\text{Ca}]_i$ , thus resembling calcium-activated chloride channels in lacrimal gland cells<sup>24</sup> and oocytes<sup>25</sup>.

### Agonist-activated nonspecific cation channels

We typically observed single channels that opened with increased frequency immediately after application of compound 48/80 or substance P (Fig. 3*a*). Also GTP- $\gamma$ -S ( $40\text{--}100 \mu\text{M}$ ) was able to induce these channels, indicating them to be G protein- or second-messenger-gated rather than receptor-activated. Perfusing cells with cAMP did not induce the opening

**Fig. 3** The characteristics of single-channel events following agonist stimulation. *a*, The cell was stimulated with substance P ( $50 \mu\text{g ml}^{-1}$ ) for the time indicated and subjected to voltage steps. Note the transient increase in inward current following agonist stimulation. The delayed chloride current, which also activated in this experiment, is truncated at depolarized voltage pulses as it exceeded 5 pA. Representative traces were taken before (1), during (2) and after (3) the inward current change, showing single-channel events detected in the whole-cell mode during hyperpolarizing episodes at  $-50 \text{ mV}$ . *b*, Current-voltage relationship for agonist-induced single channels from different cells (48/80-induced ( $\bullet$ ) and substance P-induced ( $\circ$ )). Single-channel amplitudes and the chord conductance of 44 pS pertain to standard internal and external solutions. Note that our standard external saline contains 5 mM  $\text{MgCl}_2$ . In a more 'conventional' saline (2 mM  $\text{MgCl}_2$ ) we measured 53 pS slope conductance and in a low-divalent saline (0.1 mM  $\text{CaCl}_2$ , 0.1 mM  $\text{MgCl}_2$ ) we measured 67 pS. *c*, Single channels as in (*a*), but activated by compound 48/80 and recorded at  $-60 \text{ mV}$  in external saline of the following composition (in mM):  $\text{BaCl}_2$  95, HEPES-NaOH 10, glucose 11, pH 7.2.



of the cation channels. They had a reversal potential near 0 mV, suggesting cations as current carriers, and a linear current-voltage relation between  $-20$  and  $-60 \text{ mV}$ , with a conductance of  $\sim 50 \text{ pS}$  (Fig. 3*b*). When the standard bath saline was exchanged for isotonic barium solution, channel amplitude was reduced to 16 pS (Fig. 3*c*), suggesting that the channels are permeable to divalent cations. The single-channel current increased when external divalent cations were removed, as with other nonspecific calcium-permeable channels<sup>26,27</sup>. These channels were observed following stimulation when  $[\text{Ca}]_i$  was chelated to virtually zero (1–2 mM EGTA), so they are not activated by the secretagogue-induced increase in  $[\text{Ca}]_i$ . In fact, the opening frequency of these channels was drastically reduced as  $[\text{Ca}]_i$  increased above  $0.5 \mu\text{M}$ , similar to  $\text{IP}_3$ -gated channels in lymphocytes<sup>7</sup>.

In view of the apparent similarity to  $\text{IP}_3$ -activated cation channels in lymphocytes, we tested if the 50-pS channels are activated by  $\text{IP}_3$ . With a wide range of  $\text{IP}_3$  concentrations (0.5–10  $\mu\text{M}$ ), however, we found no significant increase in channel activity over unstimulated control cells. Without external stimulation, the frequency of single-channel openings was low and tended to increase slightly with time, both in the presence and absence of  $\text{IP}_3$ .

Given the properties of the 50-pS cation channels, they should contribute to calcium influx at hyperpolarized voltages, but as their whole-cell currents rarely exceeded 10 pA, they cannot entirely account for the observed changes in  $[\text{Ca}]_i$ . This is because currents through nonspecific channels should behave like leak currents (either spontaneous or experimentally induced), which we find must be in the range of 10–20 pA in order to produce increases in  $[\text{Ca}]_i$ . Also, there was no tight correlation between the activity of the 50-pS channel and changes in  $[\text{Ca}]_i$ . Increases in  $[\text{Ca}]_i$  were not present in all cells that showed substantial channel activity (currents up to 4 pA). Conversely, we encountered cells in which there was little activity of the 50-pS cation channels, but normal development of hyperpolarization-driven increases in  $[\text{Ca}]_i$ . It is inferred that changes in  $[\text{Ca}]_i$  may be more closely associated with a small, smooth inward current that can be discerned under favourable circumstances (see next section).

### Calcium influx induced by $\text{IP}_3$

The agonist-induced changes in  $[\text{Ca}]_i$  could be mimicked by  $\text{IP}_3$  applied in the pipette solution (Figs 1*c* and 4*a*). Whereas the mobilization of calcium from internal stores was extremely fast (at concentrations larger than 1  $\mu\text{M}$ ), such that quite often

only the trailing edge of the calcium transient was seen, the voltage-dependent changes in  $[\text{Ca}]_i$  developed rather slowly (Fig. 4*a*) and were abolished upon removal of extracellular calcium (Fig. 4*b*). The slow onset of voltage-dependent changes in  $[\text{Ca}]_i$  may be due to slow gating kinetics of the calcium influx mechanism or to build-up of a required co-factor.  $\text{IP}_4$  has been suggested to induce calcium influx by itself or synergistically with  $\text{IP}_3$ <sup>8,9</sup>. We cannot rule out a possible role for  $\text{IP}_4$  in supporting  $\text{IP}_3$ -induced calcium influx, since it may be generated from  $\text{IP}_3$  by the  $\text{IP}_3$ -kinase. However, perfusing cells with 10  $\mu\text{M}$   $\text{IP}_4$  did not cause  $\text{IP}_3$ -like changes in  $[\text{Ca}]_i$  ( $n=9$ ) and application of  $\text{IP}_4$  together with  $\text{IP}_3$  was not noticeably different from application of  $\text{IP}_3$  alone. That is, the response pattern of a low concentration of  $\text{IP}_3$  (0.5  $\mu\text{M}$ ), which caused hyperpolarization-driven increases of  $[\text{Ca}]_i$  with variable delays in about 50% of the cells, was not enhanced in any way by the additional presence of a high concentration of  $\text{IP}_4$  (10  $\mu\text{M}$ ) in 10 paired experiments.

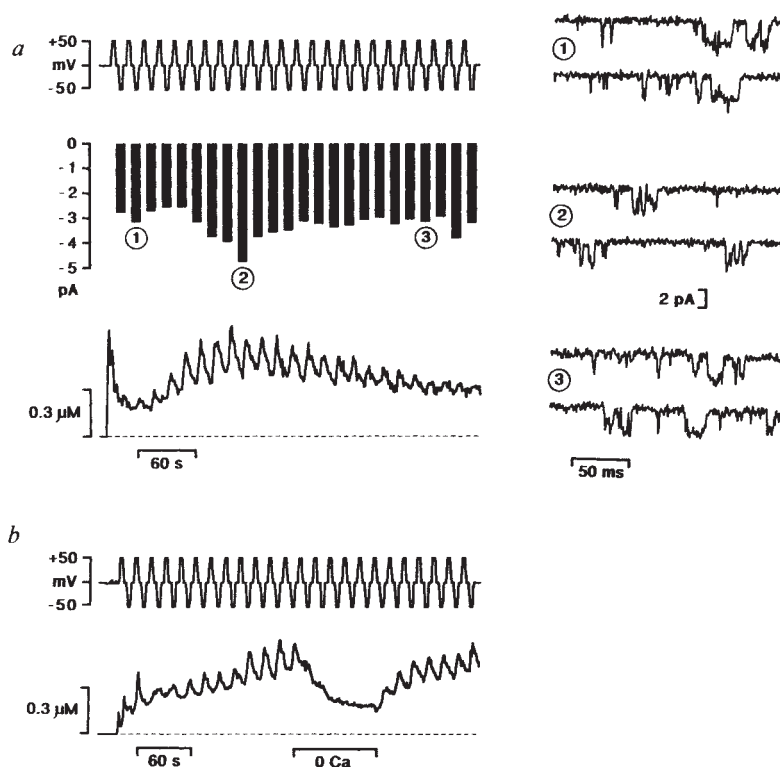
We rarely observed clear and unequivocal inward currents following application of  $\text{IP}_3$ . This may not speak against  $\text{IP}_3$ -activated currents, because the amplitude of a calcium-specific current necessary for an effect on  $[\text{Ca}]_i$  is in the range of only 1–2 pA (ref. 18), as compared to 10–20 pA for a non-specific current. Often, the delayed chloride current, activity of the large cation channel, or an initial or developing leak between the pipette and the plasma membrane prevented detection of such small  $\text{IP}_3$ -induced currents. On favourable occasions, however, where chloride currents were blocked by DIDS and the leak current was small and unaltered after the effects of  $\text{IP}_3$  had subsided, we observed a small increase in inward current correlated with the phase of voltage-dependent calcium entry (see bar graph in Fig. 4*a*). In the cell of Fig. 4*a*, there was no correlation between  $[\text{Ca}]_i$  and the frequency of opening of the 50-pS cation channels (previous section) that were riding on top of the  $\text{IP}_3$ -induced current. Representative traces that show single-channel activity before, during and after the phase of voltage-dependent phase of calcium entry are provided in Fig. 4*a*. Thus, the calcium influx seemed related to a 1–2 pA, smooth inward current not associated with visible channel events, rather than to the activity of 50-pS channels.

### Functional significance for secretion

Stimulation of rat peritoneal mast cells by secretagogues initiates complex mechanisms for the regulation of  $[\text{Ca}]_i$ . Our results suggest that substance P and compound 48/80 stimulate PI breakdown, liberating  $\text{IP}_3$ , which releases internal calcium and



**Fig. 4** Effects of  $\text{IP}_3$  on inward current and single-channel activity. *a*, The cell was perfused with standard internal solution containing  $4\text{ }\mu\text{M}$   $\text{IP}_3$  and voltage-clamped to three different potentials. Inward current for hyperpolarizing voltage pulses are illustrated as a bar graph. Note the temporal correlation between inward current amplitude and the resulting voltage-dependent changes in  $[\text{Ca}]_i$ . In addition, representative traces showing single-channel events before (1), during (2) and after (3) the voltage-dependent plateau phase of  $[\text{Ca}]_i$  were taken during hyperpolarizing episodes. In this experiment,  $10\text{ }\mu\text{M}$  DIDS was present in internal and external solutions to reduce the contribution of the delayed chloride current. *b*, Experimental protocol as in (*a*), but with  $0.5\text{ }\mu\text{M}$   $\text{IP}_3$  and no external or internal DIDS. At the time indicated, the standard bath solution was replaced by nominally calcium-free saline to illustrate the dependence of  $\text{IP}_3$ -induced changes in  $[\text{Ca}]_i$  on external calcium.



produces an initial transient increase in  $[\text{Ca}]_i$ . Possibly in conjunction with another co-factor,  $\text{IP}_3$  also activates a pathway for external calcium entry that does not produce large inward currents. In addition, calcium may permeate through non-specific cation channels that are activated by an as yet unidentified mechanism. These influx pathways represent the mechanism for the sustained phase of  $[\text{Ca}]_i$ .

In current-clamp experiments, we observed membrane hyperpolarization to about  $-40\text{ mV}$  following activation of the chloride current, with a concomitant rise in  $[\text{Ca}]_i$ . This suggests that activation of the chloride current in intact cells will increase calcium influx by providing electrical driving force. In the absence of chloride conductance, the membrane potential after secretagogue stimulation would approach the reversal potential for the agonist-activated conductances, that is positive values for a calcium-specific mechanism or values close to  $0\text{ mV}$  for a non-selective cation conductance. But the large delayed chloride current, the dominant conductance in stimulated cells, will bring the membrane potential near the chloride equilibrium potential,  $E_{\text{Cl}}$ . We do not know the exact value of  $E_{\text{Cl}}$  in the intact cell. In other non-excitable cells, however, where  $E_{\text{Cl}}$  can be measured more accurately, it is  $-30$  to  $-40\text{ mV}$  (refs 28, 29), which is the range of potentials in which we observed hyperpolarization-driven calcium increases. Thus, activation of the chloride current would likely clamp the potential sufficiently negative to support calcium influx.

The role of cAMP in mast-cell secretion has been the subject of much research but remains a matter of controversy and confusion. We suggest a stimulatory role for cAMP by virtue of providing electrical driving force for calcium influx. Most pharmacological evidence, however, points towards an inhibitory action, since drugs that are known to elevate cAMP suppress secretion<sup>30</sup>. Patch-clamp experiments using capacitance measurement in fact confirm such an inhibitory mechanism of cAMP on secretion at fixed membrane voltage and  $[\text{Ca}]_i$  (unpublished observations). The discrepancies may be resolved by differential effects of cAMP on the mechanisms that govern secretion and on calcium influx. The balance between inhibitory actions on secretion and facilitatory effects on calcium influx

may determine the resulting secretory response and may explain some of the controversial reports on the role of cAMP in mast-cell secretion. In one study, however, the inhibitory actions of cAMP on secretion have been attributed to its ability to suppress antigen-induced calcium influx, based on tracer-flux studies in the presence of dibutyryl-cAMP and forskolin<sup>12</sup>. At present we have no convincing explanation for this discrepancy.

We have identified two conductance pathways for calcium entry, one of which can be mimicked by  $\text{IP}_3$ . Single channels induced by  $\text{IP}_3$  have long been searched for, but so far only one study has reported the presence of calcium-specific single channels activated by micromolar concentrations of  $\text{IP}_3$  in excised membrane patches<sup>7</sup>. We have been unable to find single-channel events induced by  $\text{IP}_3$ . Instead, we have found a very small current associated with  $\text{IP}_3$ -induced calcium influx. This current could not be resolved into single-channel events, indicating a small single-channel conductance or a different mechanism of Ca entry<sup>31</sup>. Small calcium-specific channels have been observed in reconstituted membranes from mast cells and lymphocytes<sup>32,33</sup>.

In addition, we found increased activity of 50-pS cation channels following stimulation with secretagogues, but these channels were not activated by  $\text{IP}_3$ . It is likely that a second messenger is involved in the gating of these channels, because they are also activated by GTP- $\gamma\text{-S}$  and do not show the brisk onset and decay of activity with stimulation that is typical for directly ligand-activated channels. Alternatively, these channels may be directly gated by G proteins. Their activation even at low intracellular calcium ( $1\text{--}2\text{ mM}$  EGTA) and the inability of  $\text{IP}_3$  to activate them distinguishes these channels from previously described second-messenger-gated cation conductances in neutrophils<sup>4</sup>, where  $[\text{Ca}]_i$  activates channels, and in lymphocytes<sup>7</sup>, where  $\text{IP}_3$  has been suggested to open plasma membrane channels.

Taken together, it is clear that in addition to the mobilization of calcium from internal stores, there is a prominent calcium influx in mast cells following stimulation. Optimal conditions for influx are provided by interactive and synergistic actions of the second messengers  $\text{IP}_3$  and cAMP. Although a rise of  $[\text{Ca}]_i$

into the range of that observed under physiological conditions is not sufficient by itself to induce secretion in mast cells<sup>34,35</sup>, there is general agreement that increased  $[Ca]_i$  can dramatically enhance secretion in the presence of an additional signal<sup>36,37</sup>. In particular, calcium sensitivity of the secretory mechanism

increases some 20–40 s after a stimulus<sup>18</sup>. Unlike the fast calcium transient, which occurs too early to contribute significantly to secretion, the plateau phase caused by calcium influx is ideally timed to influence the secretory response during this period of heightened sensitivity.

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## LETTERS TO NATURE

### Probable optical counterpart of the eclipsing millisecond pulsar system, 1957+20

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Following the discovery of an eclipsing, millisecond pulsar 1957+20 (ref. 1) we conducted a series of optical observations to identify the optical counterpart of the system. We report the detection of a probably variable object (hereafter star X) with a mean apparent V magnitude of  $\sim 20$  within the astrometric error circle of the equatorial radio position of the pulsar. Star X appears fainter at orbital phase 0.21, the onset of the pulsar eclipse, compared with the quadrature position, suggesting that it is probably the optical counterpart. We argue that the secondary heated by the pulsar wind and not the pulsar itself makes the dominant contribution to the visible radiation from this system. The absolute magnitude of star X is  $M_v \approx 10.5$ , assuming a distance of  $\sim 0.8$  kpc derived from the dispersion measure. The optical luminosity of the counterpart directly measures the fraction of the pulsar wind power responsible for heating the secondary. Future optical observations may be able to constrain the nature of the pulsar wind.

The equatorial positions of all stars brighter than  $V \approx 15$  and within  $0.2^\circ$  of the pulsar radio position (hereafter the secondary stars) were extracted from the *Guide Star Catalog* of the Hubble Space Telescope; the r.m.s. accuracy of the positions of the secondary stars is believed to be 0.33 arcsec with respect to stars in the AGK3 catalogue. We digitized a small area centred on the pulsar radio position on a glass copy of the appropriate Palomar Observatory Sky Survey red plate. Using the positions of the secondary stars, the equatorial positions of a series of

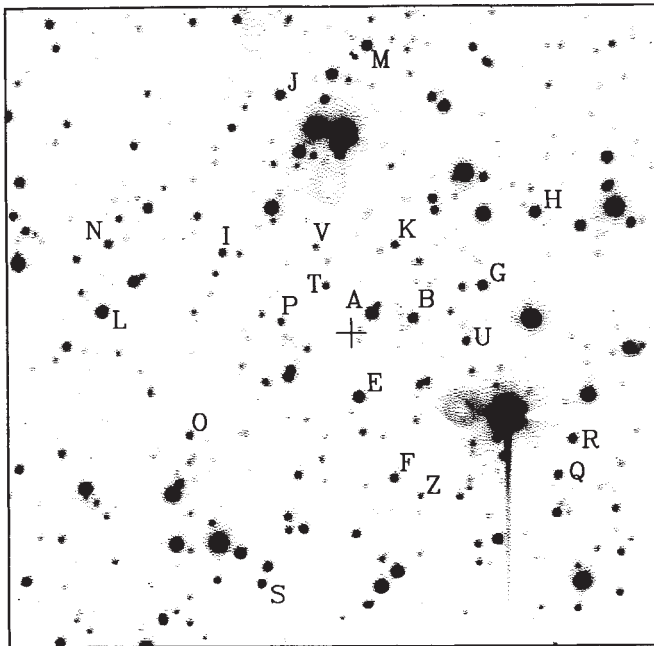
fainter stars ( $V \leq 19$ , hereafter the tertiary stars, presented in Table 1) were obtained in the usual manner. The overall r.m.s. uncertainty (systematic and random) of the positions of the tertiary stars was found to be 0.5 arcsec in each coordinate.

During the night of 1 May 1988 UT, Dr J. Cohen kindly obtained for us some exposures of the field, using a Texas Instruments 800×800 charge-coupled device (CCD) detectors at the Cassegrain focus of the Palomar 60-in telescope. The average of these exposures with the tertiary stars marked is shown in Fig. 1. Using the positions of the tertiary stars to define the reference system we derived the pixel corresponding to the radio equatorial position of the pulsar obtained at the Very Large Array (VLA); the VLA observations will be reported elsewhere. The equatorial positions of three stars (labelled C, D and X) that we considered, from their proximity to the VLA position, to be plausible optical counterparts were also derived from the same reference system.

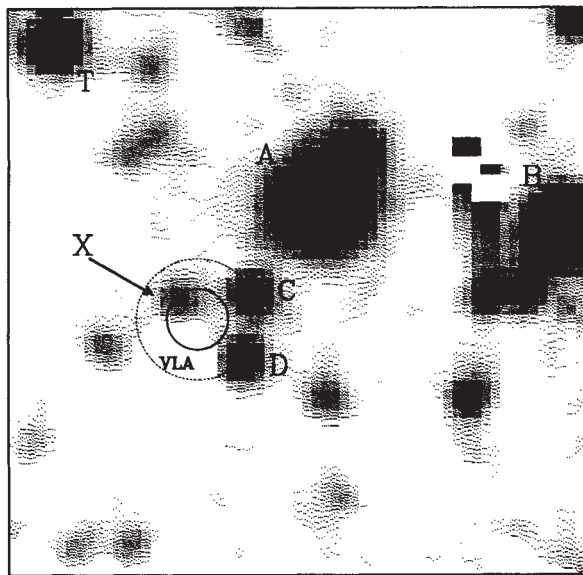
The positions of C, D and X (listed in Table 1) are uncertain by 0.6 arcsec (1 standard deviation) in each axis, significantly smaller than the 1.5 arcsec error of the radio position. As can be seen from Fig. 2, on this positional coincidence alone, star X appears to be the best candidate for the optical counterpart.

On the night of 9 May 1988 UT, we obtained a series of CCD images at the 60-in telescope in the Gunn g and r bands to look for any optical variability of star X, such as orbital modulation. Several images were obtained using a camera at the Cassegrain focus. A Texas Instruments 800×800 CCD detector with re-imaging optics (1 pixel = 1.2 arcsec) was used. Unfortunately, a large fraction of our images were saturated by the bright moonlit sky and other effects, leaving us three usable g (exposures 360 s each) and three usable r (exposures 360, 200 and 60 s) images. The g band images were spaced closely, and provide little coverage: there is no indication of significant variability in the span of  $\sim 80$  min, covering the phase range  $-0.1$  to  $0.04$ . Our definition of the phases follows that of Fruchter *et al.*<sup>1</sup>: at phase 0.25, the pulsar is farthest from the observer with the secondary in between. The r band images we obtained were at UT 9:49, 10:05 and 12:00, corresponding to phases  $-0.03$ ,  $0.0$  and  $0.21$ .





**Fig. 1** Low-contrast print of a  $V+I$  stack CCD image of the PSR 1957+20 field, 3.25 arc min on a side. North is to the top, east to the left. The effective pixel scale is 0.49 arcsec per pixel. The tertiary position standards from Table 1 are marked. The cross denotes the pulsar VLA position.



**Fig. 2** High-contrast zoom-in from the CCD image shown in Fig. 1, 30 arcsec on a side. Tertiary stars A, B and E and the candidate objects C, D and X are marked. The solid and dotted circles denote the VLA radio position, and correspond to the  $1\sigma$  and  $2\sigma$  combined errors, with radii of 1.6 and 3.2 arcsec respectively.

**Table 1** Astrometry in the PSR 1957+20 field

| Object                   | Right ascension (1950) | Declination (1950) |
|--------------------------|------------------------|--------------------|
| <b>Tertiary stars</b>    |                        |                    |
| Star A                   | 19 h 57 min 24.48 s    | +20° 40' 4.1"      |
| Star B                   | 19 h 57 min 23.61 s    | +20° 40' 2.5"      |
| Star E                   | 19 h 57 min 24.76 s    | +20° 39' 39.3"     |
| Star F                   | 19 h 57 min 24.01 s    | +20° 39' 14.1"     |
| Star G                   | 19 h 57 min 22.11 s    | +20° 40' 12.4"     |
| Star H                   | 19 h 57 min 21.01 s    | +20° 40' 35.0"     |
| Star I                   | 19 h 57 min 27.66 s    | +20° 40' 22.2"     |
| Star J                   | 19 h 57 min 26.46 s    | +20° 41' 11.7"     |
| Star K                   | 19 h 57 min 24.01 s    | +20° 40' 24.9"     |
| Star L                   | 19 h 57 min 30.26 s    | +20° 40' 4.3"      |
| Star M                   | 19 h 57 min 24.61 s    | +20° 41' 25.9"     |
| Star N                   | 19 h 57 min 30.11 s    | +20° 40' 25.2"     |
| Star O                   | 19 h 57 min 28.38 s    | +20° 39' 27.1"     |
| Star Q                   | 19 h 57 min 20.50 s    | +20° 39' 15.1"     |
| Star R                   | 19 h 57 min 20.20 s    | +20° 39' 26.2"     |
| Star S                   | 19 h 57 min 26.76 s    | +20° 38' 41.9"     |
| Star T                   | 19 h 57 min 25.48 s    | +20° 40' 12.5"     |
| Star U                   | 19 h 57 min 22.45 s    | +20° 39' 55.8"     |
| Star V                   | 19 h 57 min 25.70 s    | +20° 40' 24.2"     |
| Star Z                   | 19 h 57 min 23.44 s    | +20° 39' 8.6"      |
| <b>Candidate objects</b> |                        |                    |
| Star C                   | 19 h 57 min 24.73 s    | +20° 39' 59.4"     |
| Star D                   | 19 h 57 min 24.73 s    | +20° 39' 56.2"     |
| Star X                   | 19 h 57 min 24.99 s    | +20° 39' 59.0"     |
| <b>Pulsar (VLA)</b>      |                        |                    |
| PSR 1957+20              | 19 h 57 min 24.94      | +20° 39' 58.0"     |

The estimated errors for the tertiary stars A–Z are 0.5" in both coordinates; for stars C, D and X, 0.6". The radio position (see text) is uncertain by  $\sim 1.5''$  (error radius).

esting object. In particular star X is dimmer at pulsar eclipse which is indeed exactly what is expected since at this phase the heated side of the secondary is away from the observer.

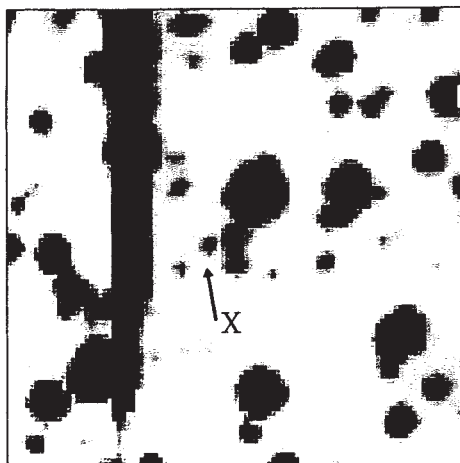
In order to quantify this variation, we performed digital stellar photometry on the CCD frames, using DAOPHOT, a stellar photometry package<sup>2</sup>. Sixteen relatively isolated unsaturated stars, typically 1–2 mag brighter than star X, were used to define the 'photometric baseline' in each frame; the stability of this baseline was found to be  $\sim 0.01$  mag. Stars C, D and X were measured against this baseline. Stars C and D stayed constant within their measurement errors ( $\sim 0.1$ – $0.2$  mag), but star X showed a variation in the three  $r$  frames: at phases  $-0.03$ ,  $0.00$  and  $0.21$ . The magnitude difference  $\Delta r$  (star X–baseline) was  $0.78 \pm 0.08$ ,  $0.99 \pm 0.18$  and  $1.64 \pm 0.51$  respectively. The change is marginally significant ( $\sim 1.6$  standard deviations, or  $\sim 90\%$  confidence level, between the first and the last frame), but it goes in the expected way.

We calibrated our photometry using short exposures of the standard star Ross 711 (ref. 3). The candidate object star X has  $g = 20.3$  and  $r = 19.7$ , with uncertainty of  $\sim 0.1$  mag, at the quadrature phase. We see no indications for a variation in colour in our short time baseline. The observed  $(g-r)$  colour of star X corresponds to a late K-type dwarf (photospheric temperature  $\sim 4,000$  K). Because of the interstellar extinction on the line of sight, the emitted colour at  $\sim 0.8$ – $1$  kpc distance would be a couple of tenths of a magnitude bluer, and the inferred photospheric temperature higher, perhaps 5,000 K. Using the transformations given by Kent<sup>3</sup>, we estimate the observed  $V$  magnitude to be  $\sim 20.0$ , and the observed  $(B-V) \approx 1.1$ . Stars C and D have  $r$  magnitudes 18.8 and 19.05, and  $(g-r)$  colours of 0.5 and 0.8, respectively. These colours are quite normal for stars of this magnitude and in this direction on the sky.

The astrometric coincidence and the possible variability in the sense expected from the eclipsing suggest that star X is

The last  $r$  image was thus obtained at the start of a pulsar eclipse whereas the first was obtained at quadrature. Sections of the first and the last  $r$  image are shown in Fig. 3. There is a suggestion that star X (our candidate) was considerably fainter in the near-eclipse frame. Of the three plausible candidates, only star X shows apparent variability, already marking it to be an inter-

09:49 UT (~ Quadrature)



12:00 UT (~ Eclipse)

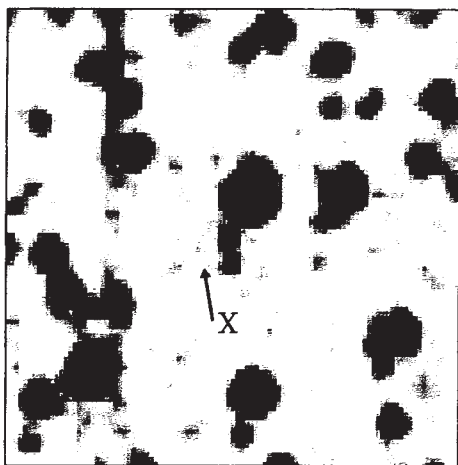


Fig. 3 Sections of the *r*-band CCD frames of the candidate field, 1 arcmin on a side, corresponding to different orbital phases as indicated. The images were scaled to the same mean signal using our stellar photometry and the image with better seeing (on the right) was convolved with a gaussian with  $\sigma = 0.55$  pixels to match the point-spread functions. Star X appears variable, in agreement with our relative photometry.

probably the optical counterpart of the system 1957+20. Clearly, further optical monitoring is needed in order to confirm our identification. Follow-up spectroscopy would be of great interest for stellar evolutionary theories.

Regardless of this uncertainty, one conclusion is firm—the counterpart of 1957+20 is not brighter than the brightest of the three candidates C, D and X: it has  $V \geq 19$ . This result alone allows us to infer that only a small fraction of the pulsar wind is converted to heat, otherwise the expected magnitude, assuming a dispersion measure distance of 0.8 kpc, reasonable extinction and a  $\dot{P}$  similar to the 1.6-ms pulsar 1937+214, would be 16 mag (ref. 4); the expected optical contribution from incoherent radiation evaluated using the formulation of Pacini and Salvati<sup>5</sup> is considerably smaller than the brightness of star X. The low luminosity of the object favours models in which the pulsar wind is predominantly in the form of low-energy  $\gamma$ -rays<sup>6</sup> or X-rays<sup>4</sup> which cause efficient ablation.

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## Time delay of the PeV gamma ray burst after the October 1985 radio flare of Cygnus X-3

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Cygnus X-3 remains a puzzling and controversial source of ultra-high-energy radiation,  $E \geq 0.1$  PeV (1 PeV =  $10^{15}$  eV). At these energies the radiation is variable<sup>1–3</sup>, with periodicity 4.8 h and a prominent peak at phase  $\sim 0.2$  during 1976–1980 and at phase  $\sim 0.6$  after 1984. There are outstanding difficulties in explaining both the phase diagram of the radiation and also the high luminosity in particles,  $L_p = 10^{40}$  erg s<sup>-1</sup>. In existing data, TeV and sometimes PeV radiation has been seen episodically; such an episode is connected with the radio flare of Cyg X-3 in October 1985, when PeV radiation with no phase structure was seen. The PeV pulse was detected<sup>5</sup> 3–5 days after the radio flare. It was suggested<sup>6</sup> that this delay could be explained by introducing a massless free gluon as an intermediary, but here I propose a more natural explanation in which gamma-photons of PeV energy are absorbed by radio radiation inside the source. After a delay, the gamma radiation emerges as the radio flux diminishes and absorption decreases.

Two groups have reported the detection of TeV and PeV gamma-radiation during the October 1985 radio flare. Gulmarg gamma-telescope detected<sup>7</sup> TeV gamma-radiation on 10 October and 12 October during the period of the large flux of radio radiation (the maximum occurred on 9 October). The detected averaged flux for  $E > 6$  TeV is  $(1.8 \pm 0.2) \times 10^{-10}$  cm<sup>-2</sup> s<sup>-1</sup>.

The Baksan EAS array was monitoring<sup>5</sup> the source during 1–20 October and detected a statistically significant signal on 14–16 October, that is, 3–5 days after the maximum of radio radiation, when radio flux became  $\sim 10$  times smaller. The detected flux is  $(4.9 \pm 1.5) \times 10^{-12}$  cm<sup>-2</sup> s<sup>-1</sup> at  $E_\gamma \geq 0.2$  PeV. The power law spectrum corresponding to these two flux values is characterized by the integral spectrum exponent  $\gamma \approx 1.0$ –1.1, which is usual for Cyg X-3.

Several days later, on 17–19 October, the source was observed

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**Table 1** Absorption lengths for two different energies

| $\alpha$ (spectral index)         | 0.1               | 0.2               | 0.3               | 0.4                  | 0.5                  | 0.8                  | 1.0                  |
|-----------------------------------|-------------------|-------------------|-------------------|----------------------|----------------------|----------------------|----------------------|
| $\kappa_\alpha$ (from equation 7) | 1.32              | 1.15              | 1.00              | 0.886                | 0.790                | 0.583                | 0.489                |
| $l_\gamma$ (0.2 PeV) cm           | $3.6 \times 10^8$ | $1.2 \times 10^9$ | $2.4 \times 10^9$ | $4.6 \times 10^9$    | $8.5 \times 10^9$    | $5.4 \times 10^{10}$ | $1.8 \times 10^{11}$ |
| $l_\gamma$ (6 TeV) cm             | $9.1 \times 10^8$ | $2.5 \times 10^9$ | $6.9 \times 10^9$ | $1.9 \times 10^{10}$ | $4.9 \times 10^{10}$ | $8.9 \times 10^{11}$ | $6.0 \times 10^{12}$ |

with Fly's Eye detector<sup>8</sup> without the detection of significant enhancement of the flux above the background. The derived upper limits at  $E_\gamma \geq 0.1$  PeV,  $2.2 \times 10^{-12} \text{ cm}^{-2} \text{ s}^{-1}$  and  $1.3 \times 10^{-12} \text{ cm}^{-2} \text{ s}^{-1}$ , for the phases 0.2–0.3 and 0.6–0.7 respectively, are consistent with the Baksan results during this period.

Radio flux measured at  $\lambda = 11.1$  cm reached the maximum value  $j_\nu \approx 18$  Jy on 10 October. It is known from the other burst observations<sup>9–10</sup> that both radio and infrared radiations display the 4.8 h periodicity with a modulation depth of  $k \approx 10\%$ . It indicates that at least some part of this radiation is produced within the region of a binary separation. We assume that periodic component of the radio-infrared radiation has a power-law spectrum and the energy flux can be taken as

$$j_\nu = j_{\nu_0} (\nu/\nu_0)^{-\alpha} \quad (1)$$

where  $\nu_0 = 2.7$  GHz corresponds to  $\lambda = 11.1$  cm and  $j_{\nu_0}$  is assumed to be  $\sim 1$  Jy.

The results of radio and gamma-ray observations during the flare are illustrated in Fig. 1. The Gulmarg array detected TeV gamma-radiation during the radio flare maximum (shown by arrows), and the Baksan array detected the PeV radiation several days later.

Let us now consider the  $\gamma + \gamma \rightarrow e^+ + e^-$  absorption of TeV and PeV gamma-quanta by radio and infrared radiation in the source. This absorption for Cyg X-3 has been discussed by Apparao<sup>11</sup> and Protheroe and Stanev<sup>12</sup> in another context.

The absorption length for a photon with energy  $E_\gamma$  can be calculated<sup>13</sup> as

$$l_\gamma^{-1} = \frac{4}{E_\gamma^2} \int_{m_e}^{\infty} d\omega_c \sigma(\omega_c) \omega_c^3 \int_{\omega_c/E_\gamma}^{\infty} \frac{d\omega}{\omega^3} \rho(\omega) \quad (2)$$

where  $m_e = 0.511$  MeV is the electron mass,  $\omega$  and  $\omega_c$  are the energies of the 'target' photons in the laboratory system and centre of mass system respectively,  $\rho(\omega)$  is the energy density of the target, namely the radio-infrared radiation inside the source and  $\sigma(\omega_c)$  is the  $\gamma + \gamma \rightarrow e^+ + e^-$  cross-section, which in

terms of the variable  $v = (1 - m_e^2/\omega_c^2)^{1/2}$  can be written as

$$\sigma = \frac{3}{16} \sigma_T (1 - v^2) \left\{ (3 - v^4) \ln \frac{1+v}{1-v} + 2v(v^2 - 2) \right\}, \quad (3)$$

where  $\sigma_T$  is the Thompson cross-section.

To estimate the energy density of the target photons in the source we assume that a periodic component of radio and infrared radiation originates within the modulation region  $r \leq D$ , where  $D \approx 1 \times 10^{11} (M/M_\odot)^{1/3}$  cm is the separation of the stars in the binary and  $M$  is the total mass of the system. Then for  $\rho(\omega)$ , one obtains

$$\rho(\omega) = \frac{r^2}{cD^2} j_0 \left( \frac{\omega}{\omega_0} \right)^{-\alpha} \quad (4)$$

where  $r = 13$  kpc is the distance to Cyg X-3,  $\omega_0 = hc/\lambda_0 = 1.12 \times 10^{-5}$  eV corresponds to  $\lambda_0 = 11.1$  cm and

$$j_0 = 1.5 \times 10^3 (j_{\nu_0}/1 \text{ Jy}) \text{ cm}^{-2} \text{ s}^{-1} \quad (5)$$

Finally we get from equations (2)–(5)

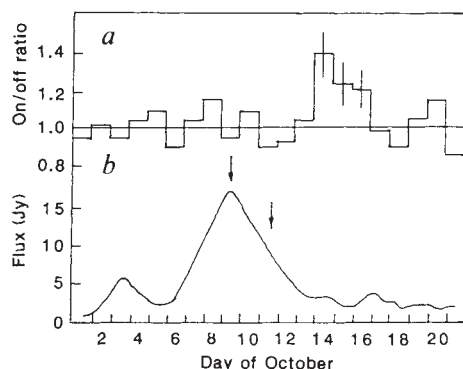
$$l_\gamma^{-1} = \frac{3}{4(2+\alpha)} \frac{r^2}{D^2} \frac{\sigma_T j_0}{c} \left( \frac{E_\gamma \omega_0}{m_e^2} \right)^\alpha \kappa_\alpha \quad (6)$$

where

$$\kappa_\alpha = \int_0^1 v dv (1 - v^2)^\alpha \left\{ (3 - v^4) \ln \frac{1+v}{1-v} + 2v(v^2 - 2) \right\} \quad (7)$$

is tabulated in Table 1. Here the absorption lengths for gamma-quanta with  $E_\gamma = 0.2$  PeV (Baksan) and  $E_\gamma = 6$  TeV (Gulmarg) are also listed. One can see that for  $\alpha > 0.4$  the former is much shorter than the latter. Therefore, PeV gamma-ray radiation can be absorbed within the source during the maximum of the radio flare, with TeV gamma-radiation freely escaping from it.

The calculations given here have an illustrative character as the spectrum of radio and infrared radiation is unknown for the flare. Moreover, there were no measurements of infrared radiation during the flare. Our basic observation is that PeV and TeV gamma quanta are absorbed by the target photons of different energies ( $2.6 \times 10^{-3}$  eV and  $8.7 \times 10^{-2}$  eV for  $E_\gamma = 0.2$  PeV and  $E_\gamma = 6$  TeV, respectively) and that the flux of the low-energy target photons can be considerably higher, thus providing the stronger absorption of PeV gamma-radiation.



**Fig. 1** The results of Cyg X-3 observation in PeV gamma-radiation<sup>5</sup> (a) and radio radiation (b). Arrows show the time of observation by Gulmarg telescope. a, Plot of on/off source ratio for PeV gamma-radiation. The bars show  $1\sigma$  standard deviation.

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# Nitrogen and helium enhancement in the progenitor of supernova 1987A

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Why the progenitor of supernova 1987A was a blue supergiant has been a fundamental question, because the occurrence of a type II supernova from such a progenitor had not been known before. Recent ultraviolet observations have provided crucial evidence that bears on this problem. Emission lines of CNO elements show that the expansion velocity of the emitting gas is  $<30 \text{ km s}^{-1}$ , and that the abundance ratios of N/C and N/O are larger than the solar ratios by factors of  $\sim 30$  and 10, respectively<sup>1,2</sup>, indicating that the ultraviolet emissions come from circumstellar material which had been processed by hydrogen burning in the interior and ejected into space when the progenitor was a red supergiant. The progenitor of SN1987A must therefore have evolved first to a red supergiant and then back to the blue. From calculations of massive star evolution, we show how the star can undergo blue-red-blue evolution. By comparing these theoretical models with the abundance information from the ultraviolet observations, we conclude that the hydrogen-rich envelope of the progenitor was as massive as  $7\text{--}11 M_{\odot}$  and almost the whole envelope was uniformly mixed, probably because of rapid rotation.

The progenitor's initial mass and the core mass are inferred, from the luminosity ( $\sim 1.3 \times 10^5 L_{\odot}$ ) of the progenitor, Sk-69 202, as  $M_1 \approx 20 M_{\odot}$  and  $M_{\text{core}} \approx 6 M_{\odot}$  (refs 3, 4). We have evolved a star of initial mass  $M_1 = 21 M_{\odot}$  from the main sequence through to carbon ignition as in our earlier work<sup>5</sup>. The initial mass fractions of He and metals were  $Y = 0.25$  and  $Z = 0.005$ . For convection, the Schwarzschild criterion was adopted. Mass loss is given as a function of  $(L, T_{\text{eff}})$  at four times the rate given in ref. 6. Other physical inputs and the evolution code are the same as in ref. 5.

Evolutionary paths in the Hertzsprung-Russell diagram for several cases of material mixing are shown in Fig. 1. During early He burning, the star is a blue supergiant. Whether the star remains blue or moves to red depends on the mass,  $M_{\text{env}}$ , and He abundance,  $Y$ , of the H-rich envelope. For given  $L$ ,  $T_{\text{eff}}$  and the mass of the evolved core  $M_{\text{core}}$ , the blue supergiant envelope solution must satisfy a certain relation between  $M_{\text{env}}$  and  $Y$  to match the core<sup>5,7</sup>. The relation obtained in ref. 5 is  $Y = 0.25$  and 0.4 for  $M_{\text{env}} = 16 M_{\odot}$  and  $10 M_{\odot}$  respectively for  $L = 1 \times 10^5 L_{\odot}$ ,  $T_{\text{eff}} = 1.6 \times 10^4 \text{ K}$ ,  $M_{\text{core}} = 5.5 M_{\odot}$  and  $Z = 0.005$ . Therefore if  $M_{\text{env}}$  were sufficiently large (almost no mass loss) for  $Y \approx 0.25$ , the star would have remained blue<sup>5</sup>, as in the models calculated in refs 8-10. But as the star loses a significant fraction of its envelope mass, it moves redward to become a red supergiant star, as in Fig. 1, because  $M_{\text{env}}$  is too small for the star to remain blue for its  $L$  and  $Y$  (ref. 5). Thus mass loss is the driving mechanism of redward evolution.

When helium is exhausted in the central region and the C+O core starts to contract, helium shell burning begins. Figure 2 shows the abundance distribution in mass fraction at this stage, where  $M_r$  denotes the mass included in a sphere of radius  $r$ . The star forms a core of  $M_{\text{core}} = 6.0 M_{\odot}$  ( $3.5 M_{\odot}$  C+O core and  $2.5 M_{\odot}$  He layer), and a  $10.3 M_{\odot}$  hydrogen-rich envelope;  $4.7 M_{\odot}$  has been lost as a wind decreasing the stellar mass from  $21 M_{\odot}$  to  $16.3 M_{\odot}$ . In the He layer, a convective shell due to He-shell burning is developing, where  $^{12}\text{C}$  is enhanced and  $^{14}\text{N}$  has been burned into  $^{18}\text{O}$  and  $^{22}\text{Ne}$ . In the H-rich envelope, He and N abundances are larger in the deeper layers; an abundance

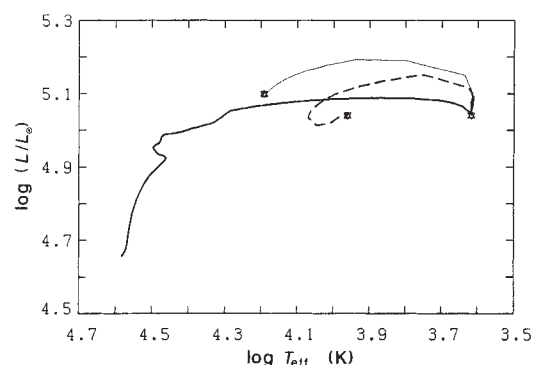


Fig. 1 Evolutionary tracks in the H-R diagram of a star, with initial mass  $M_1 = 21 M_{\odot}$  and metallicity  $Z = 0.005$ , from the main sequence through to carbon ignition (asterisk). Evolution without additional mixing is shown by the thick solid line. The dashed line assumes mixing of the whole H-rich envelope, resulting in a surface He abundance of  $Y_{\text{surf}} = 0.35$ . The thin solid line shows the evolution when  $1 M_{\odot}$  He layer is dredged up to yield  $Y_{\text{surf}} = 0.43$ . The thin solid line approaches the location of Sk-69 202 ( $\log T_{\text{eff}} \approx 4.2$  and  $\log(L/L_{\odot}) \approx 5.1$ ).

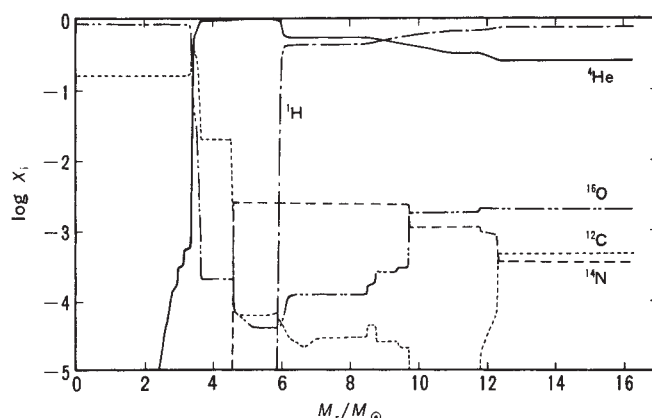


Fig. 2 Composition structure of the star of Fig. 1, where  $X_i$  denotes the mass fraction and  $M_r$  denotes the mass included in a sphere with radius  $r$ . The stage illustrated is when the C+O core forms and He-shell burning begins. The stellar mass has decreased from  $M_1 = 21 M_{\odot}$  to  $16.3 M_{\odot}$ . Abundances not shown are omitted for clarity.

gradient has formed during core H-burning. At  $6.0 M_{\odot} < M_r < 8.5 M_{\odot}$ , the materials have been homogenized in the convective shell. In the outermost layers,  $M_r > M_b = 12.3 M_{\odot}$ , the surface convection has mixed the materials. (The depth of the surface convection zone depends weakly on the mixing length  $l$ . In the above model,  $l = 1.5$  is adopted. For  $l = 0.5$ , its bottom is located at  $M_b = 11.3 M_{\odot}$  at the deepest stage.)

As He-shell burning begins, the He layer expands, causing a contraction of the H-rich envelope with the H-burning shell as a node. Whether the star returns to the blue supergiant phase depends on  $M_{\text{env}}$ ,  $Y$  and the chemical profile in the H-rich envelope. If no additional mixing occurs, the resulting decrease in the stellar radius is small; the luminosity decreases but without much change in  $T_{\text{eff}}$ . This is shown by the solid line in Fig. 1.

More extensive mixing could be induced by rapid rotation. To simulate such a case, we uniformly mix the materials from the surface down to some  $M_r$ . For deeper mixing (smaller  $M_r$ ) the resulting He abundance  $Y_{\text{surf}}$  and the abundance ratios N/C and N/O at the surface are larger, as shown by the solid lines in Fig. 3.

Evolution for several cases of deep mixing was calculated. Generally the star becomes bluer with more extensive mixing. The dashed line in Fig. 1 shows the evolution for complete



mixing of the whole H-rich envelope (down to  $M_r = 6 M_\odot$ ), yielding  $Y_{\text{surf}} = 0.37$ . The star tends to make a loop in the H-R diagram and ignites carbon when  $T_{\text{eff}} \approx 10^4$  K. This may be consistent with the value  $\log T_{\text{eff}} = 4.2$  of Sk-69 202, although the model is a little too red. If the He layer is dredged up,  $Y_{\text{surf}}$  would be larger. Evolution along the thin solid line of Fig. 1 is obtained for  $Y = 0.43$ , which simulates a He enhancement due to dredge-up of  $1 M_\odot$  of the helium layer. The star then shrinks and returns near to the location of Sk-69 202 in the H-R diagram. This dependence of the evolution on  $Y$  can also be understood from the existence or non-existence of a blue supergiant envelope solution in the plane of  $(M_{\text{env}}, Y_{\text{surf}})$ . A blue supergiant envelope near Sk-69 202 should have  $Y_{\text{surf}} \approx 0.4$  for  $M_{\text{env}} \approx 10 M_\odot$  (ref. 5).

For the star to undergo blueward excursion to the location of Sk-69 202, mixing of almost the whole H-rich envelope must have occurred, and dredge-up of part of the He layer would also have been necessary. Such large-scale mixing could not have been achieved convectively because the surface convection zone is too shallow even allowing for uncertainties in convection theory (Fig. 2). Rapid rotation, on the other hand, may induce such mixing<sup>11</sup>.

This blueward evolution occurs more easily for a lower metallicity envelope ( $Z < 0.01$ )<sup>5</sup>. An envelope with larger  $Z$  has larger opacity and to compensate, the blue supergiant envelope solution has a larger  $M_{\text{env}}$  or  $Y$  for a given  $L$ . In other words, a greater enhancement of  $Y$  is required for given  $M_{\text{env}}$  if  $Z$  is larger, so evolution from red to blue is more likely to occur for smaller  $Z$ .

From the International Ultraviolet Explorer (IUE) observations the surface abundances ratios are estimated as  $N/C = 7.8 \pm 4$  and  $N/O = 1.6 \pm 0.8$  (ref. 2). Figure 2 shows that nitrogen abundance at the surface has been enhanced to  $N/C = 0.68$  ( $X(N)/X(C) = 0.8$  by mass) because of mixing in the surface convection zone. This is 2.5 times larger than the solar ratio but smaller than that observed by a factor of  $\sim 10$ . However, more extensive mixing is indicated by the blue-red-blue evolution of the progenitor. If this is the case, the resulting  $N/C$  and  $N/O$  ratios at the surface would be larger because the materials in the deeper layers are more N-rich. For mixing from the surface down to  $M_r$ , for  $M = 16.3 M_\odot$  ( $M_{\text{env}} = 10.3 M_\odot$ ), Fig. 3 shows the surface abundance ratios as a function of  $M_r$ . The curves extend to the left of the dash-dotted line at  $M_r = 6 M_\odot$ , corresponding to a dredge-up of the  $\Delta M_{\text{core}}$  He layer. The ratio  $N/C$  increases with increasing  $\Delta M_{\text{core}}$  but starts to decrease for  $\Delta M_{\text{core}} > 1.8 M_\odot$ . Material in such deep layers has been processed by convective He-shell burning that enhances  $^{12}\text{C}$  and converts  $^{14}\text{N}$  into  $^{18}\text{O}$  and  $^{22}\text{Ne}$ . This implies that the circumstellar materials hardly contain the He-burning products. If the suggested enhancement of s-process elements (Sr and Ba)<sup>12</sup> is due to the dredge-up of the material in the convective He-burning shell, such mixing must have occurred during explosion.

Comparison between the theoretical curves and the observations in Fig. 3 indicates that mixing down to at least  $M_r = 7 M_\odot$  is required to explain the observed  $N/C$  ratio. Complete mixing in the entire H-rich envelope and a dredge-up of  $\Delta M_{\text{core}} (< 1.8 M_\odot)$  are certainly consistent with observations. For such deep mixing, the  $N/O$  ratio agrees with the observed ratio, so the observed nitrogen overabundance in the circumstellar matter can be explained by the evolution of a progenitor that experienced blue-red-blue evolution due to enhanced  $Y_{\text{surf}}$ .

The surface  $N/C$  and  $N/O$  ratios depend on  $M_{\text{env}}$  (or  $M$ ) when the mixing occurs. If the stellar mass has decreased to  $M = 13 M_\odot$  ( $M_{\text{env}} = 7 M_\odot$ ), the abundances are given by the dashed lines in Fig. 3. Mixing from the surface to  $M_r = 8.5$ – $10.5 M_\odot$  is consistent with observations but mixing down to  $M_r < 8.5 M_\odot$  (including whole envelope mixing) makes  $N/C$  too high. On the other hand, evolutionary calculations show that mixing of the whole envelope is required for the star to come to the location of Sk-69 202 in the H-R diagram. If mixing is less extensive,

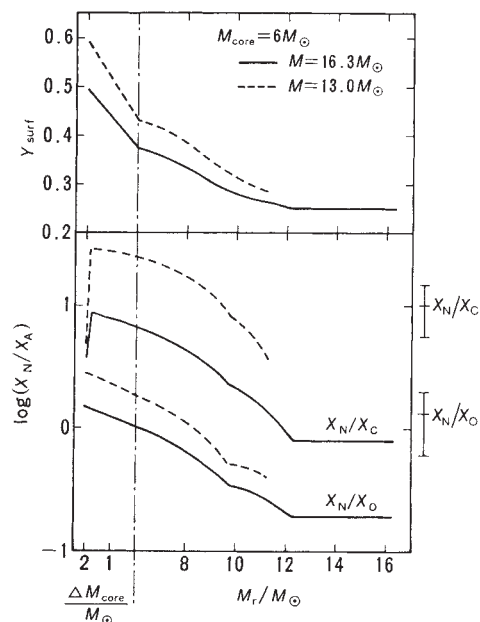


Fig. 3 Surface abundances when the envelope from the surface ( $M_r = 16.3 M_\odot$ ) down to  $M_r$  is assumed to be uniformly mixed. For  $M_r > 12.3 M_\odot$  the abundance is constant because the surface convection zone has mixed the material down to  $M_r = 12.3 M_\odot$ . The lines extended to the left of  $M_r = 6 M_\odot$  (indicated by the dash-dotted line) correspond to a dredge-up of the  $\Delta M_{\text{core}}$  He layer. If the stellar mass has decreased to  $M = 13 M_\odot$  ( $M_{\text{env}} = 7 M_\odot$ ) when mixing occurs, abundances are given by the dashed lines. The observed abundance ratios in mass fraction are indicated with error bars.

the resulting enhancement of  $Y_{\text{surf}}$  is too small for the star with  $M_{\text{env}} = 7 M_\odot$  to become sufficiently blue at carbon ignition. This implies that the progenitor's envelope should be more massive than at least  $7 M_\odot$ .

To generalize this argument, let us assume that the original composition structure of any envelope for  $M_{\text{core}} = 6 M_\odot$  is similar to Fig. 2 and that the whole H-rich envelope is uniformly mixed. Then the  $N/C$  ratio is consistent with observations only for  $7.5 M_\odot < M_{\text{env}} < 11 M_\odot$ . For smaller  $M_{\text{env}}$ , the  $N/C$  ratio is too large and vice versa. The  $N/O$  ratio is less restrictive. For a star with  $M_i = 18 M_\odot$  which forms a  $4.9 M_\odot$  He core, we find  $7 M_\odot < M_{\text{env}} < 9 M_\odot$ . The constraint on  $M_{\text{env}}$  depends on the total amount of CNO elements in the H-rich envelope, and so does not depend much on the composition structure in the semi-convective region as far as mixing of the whole envelope occurs. If dredge-up of the He layer is taken into account, the required range of  $M_{\text{env}}$  shifts to the slightly more massive side than above.

Our massive envelope model is distinct from the models obtained by Maeder<sup>13</sup> and Wood and Faulkner<sup>14</sup>, where  $M_{\text{env}} \ll 1 M_\odot$ . It has been shown, for example, that the theoretical light curve for  $M_{\text{env}} = 7$ – $11 M_\odot$  agrees well with the observation whereas the light curve for a thinner envelope reaches a maximum too early<sup>4,15,16</sup>. A thick envelope is also indicated from the analysis of optical observations<sup>17,18</sup>.

Another test for evolutionary models is the lifetime of the red supergiant phase and the amount of circumstellar matter lost from the star during it. The evolutionary timescale at each location in the H-R diagram—the change in stellar radius—is sensitive to the composition structure and  $M_{\text{env}}$ . Comparison with the observations including number ratio between the blue and red supergiants<sup>13</sup> would provide useful information on the timescale of mixing in the semi-convection zone and the mass-loss rate at various locations in the H-R diagram. This will be discussed in detail in a separate paper.

Further information on the evolutionary model could be obtained from the timescale of the envelope contraction from red to blue. In our model, it takes 18,000 yr, 10,000 yr and 6,000 yr for stars of  $M_1$  ( $M_{\text{core}}$ ) = 18  $M_{\odot}$  (4.9  $M_{\odot}$ ), 20  $M_{\odot}$  (5.5  $M_{\odot}$ ) and 21  $M_{\odot}$  (6.0  $M_{\odot}$ ), respectively. (The timescale from carbon burning through collapse is  $\sim 500$  yr for  $M_{\text{core}} = 6 M_{\odot}$  (ref. 19).) These timescales should be compared with the distances from the supernova to the dense circumstellar shells which will be estimated from the ultraviolet<sup>17</sup>, infrared<sup>20</sup> and soft X-ray<sup>21</sup> observations.

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Ultraviolet observations have provided clear evidence that the progenitor experienced blue-red-blue evolution and lost N-rich material, which can be explained by a theoretical model in which the progenitor's H-rich envelope was as massive as  $M_{\text{env}} = 7$ –11  $M_{\odot}$  and had been almost uniformly mixed. Such extensive mixing may be induced by rapid rotation<sup>22,11</sup> but investigations into the effects of magnetic field and convective overshooting are also needed. Useful constraints on the mixing mechanism could be obtained from the observed He and CNO anomalies in the blue supergiant atmospheres<sup>23</sup>.

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## A new high- $T_c$ $\text{TlBa}_2\text{Ca}_3\text{Cu}_4\text{O}_{11}$ superconductor with $T_c > 120$ K

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Three superconducting compounds are known at present in the  $\text{Tl-Ba-Ca-Cu-O}$  system:  $\text{Tl}_2\text{Ba}_2\text{CuO}_{6+y}$ ,  $\text{Tl}_2\text{Ba}_2\text{CaCu}_2\text{O}_{8+y}$  and  $\text{Tl}_2\text{Ba}_2\text{Ca}_2\text{Cu}_3\text{O}_{10+y}$ , known by their cation numbers as '2201', '2212' and '2223', respectively<sup>1–3</sup>. The transition temperature  $T_c$  increases in this series, from 80 to 110 to 120 K, as the number of  $\text{CuO}_2$  layers in the unit cell increases from one to three. Here we report the synthesis and characterization of a new  $\text{Tl-Ba-Ca-Cu-O}$  superconductor which differs from these three in having only a single thallium layer (instead of two) per unit cell, but which has four  $\text{CuO}_2$  layers. The composition of the phase is  $\text{TlBa}_2\text{Ca}_3\text{Cu}_4\text{O}_{11}$  (1234) and its structure is tetragonal, with lattice constants  $a = 3.85 \text{ \AA}$  and  $c = 19.1 \text{ \AA}$ .

Sample preparation was carried out using a furnace in a draft chamber under flowing oxygen. Because of the toxicity of thallium compounds and the high reactivity of Ca and Ba oxides with  $\text{CO}_2$  a glovebox was used. Sample composition was determined using electron probe micro-analysis and an inductively coupled plasma spectrometer. Oxygen content was measured by non-dispersive infrared inert gas fusion method using a Horiba EMGA-2800. To calibrate the composition analysis, 2212 and 2223 samples and  $\text{YBa}_2\text{Cu}_3\text{O}_{6.95}$  samples with a single phase were used as standards. The error involved in this technique was less than 3%.

The structure was determined by X-ray powder diffraction with a Cu source, electron microscopy and electron diffraction. The  $T_c$  of the sample was measured resistively and magnetically using a conventional four-probe technique and a SQUID magnetometer. The onset of  $T_c$  ( $T_{c0}$ ), midpoint of  $T_c$  ( $T_c$ ) and end

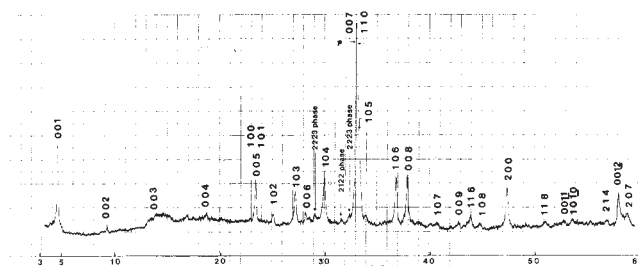


Fig. 1 X-ray powder diffraction of  $\text{Tl-Ba-Ca-Cu-O}$  with the new tetragonal phase. Lattice constants are  $a = 3.85 \text{ \AA}$  and  $c = 19.1 \text{ \AA}$ .

point of  $T_c$  ( $T_{c0}$ ) were defined as 10%, 50% and 90% drop-points of the resistance transition. The temperature was measured with a calibrated platinum resistance thermometer. The experimental error in  $T_c$  was  $\pm 0.2$  K.

Samples were prepared by firing the mixed powders of  $\text{Tl}_2\text{O}_3$ ,  $\text{CaO}$ ,  $\text{BaO}_2$  and  $\text{BaCu}_3\text{O}_4$  with a nominal composition of  $\text{Tl}_4\text{Ba}_2\text{Ca}_{n-1}\text{Cu}_n\text{O}_{2n+7}$  ( $n = 1$ –6).  $\text{BaCu}_3\text{O}_4$  powders were made by firing a mixture of  $\text{BaCO}_3$  and  $\text{CuO}$  powders in air at 1,173 K for 20 h.  $\text{CaO}$  was obtained from  $\text{CaCO}_3$  by firing at 1,173 K for 10 h. The mixed powders of starting materials were pressed into pellets of diameter 10 mm and thickness 1 mm. The pellets were fired for 10 min in flowing oxygen in a furnace that had been heated to 1,163 K. Some of the pellets were then removed and quenched in air to room temperature, the remainder being cooled to room temperature in the furnace at a cooling rate of 100  $^\circ\text{C}$  per hour.

The quenched samples are intermediate compounds having almost a single phase, which is tetragonal with the so-called 2223 structure, and lattice constants of  $a = 5.446 \text{ \AA}$  and  $c = 35.65 \text{ \AA}$  for  $n = 4, 5$  and 6. The composition of the quenched sample for  $n = 4, 5$  and 6 corresponds to the composition  $\text{Tl}_{2-x}\text{Ba}_{2-y}\text{Ca}_{2+z}\text{Cu}_{3+x}\text{O}_{10+z}$  ( $0 \leq x \leq 0.4$ ;  $0 \leq y \leq 0.3$ ;  $z \approx \pm 1$ ). The composition of some samples was slightly different from that of the ideal high- $T_c$  2223 phase<sup>3,4</sup> but had the same lattice structure.

On the other hand, the furnace-cooled samples for  $n = 4$  and 6 show a new type of tetragonal structure (see Fig. 1), with lattice constants  $a = 3.85 \text{ \AA}$  and  $c = 19.1 \text{ \AA}$ ; the phase is completely different from the previously reported 2212 and 2223 phases<sup>3–5</sup>. The sample was present as almost a single phase,

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except for the small amounts of 2223 and 2212 phases shown in the X-ray diffractogram (Fig. 1). Traces of  $\text{BaCu}_3\text{O}_4$  source material and an amorphous phase were detected by electron probe microanalysis. Almost all the peaks in Fig. 1 were identified only by the Miller indices of the new tetragonal phase. The X-ray diffractogram of Fig. 1 shows a high degree of  $c$ -axis alignment. The sample powder was artificially pressed so hard on the non-reflective silicon sample plate that the  $c$ -axis of the powders aligned perpendicular to the silicon plate and the (001) lines were apparent.

The composition of the new tetragonal phase was determined as  $\text{TiBa}_2\text{Ca}_3\text{Cu}_4\text{O}_{11}$  (1234), within an experimental error of 10%. The phase has a single Ti-O layer and four Cu-O layers, with a composition low in Ti and rich in Cu when compared with previous 2223 and 2212 Ti superconductors<sup>3,4</sup>. The Ti content in the new phase is less than half that in the 2223 phase.

Electron microscopy revealed a unit cell with dimensions 19.1 Å along the  $c$ -axis and 3.9 Å along the  $a$  and  $b$  axes; diffraction patterns indicate a simple tetragonal structure. The observed  $c$ -lattice parameter is consistent with the  $c$ -axis rule of  $c = 6.3 + 3.2n = 19.1$  Å ( $n = 4$ ) for the single Ti-O layer compound  $\text{TiBa}_2\text{Ca}_{n-1}\text{Cu}_n\text{O}_{2n+3}$ , but inconsistent with the  $c$ -axis rule of  $c/2 = 8.2 + 3.2n$  for the double Ti-O layer compound  $(\text{TiBa}_2\text{Ca}_{n-1}\text{Cu}_n\text{O}_{2n+4})_2$  (ref. 5).

Figure 2 shows the temperature dependence of the resistance for the present low-Ti sample with the 1234 phase (a) in comparison with a quenched intermediate high-Ti sample with the 2223 phase (b). The  $T_{c0}$ ,  $T_c$  and  $T_{c*}$  values of the former sample are 126.6, 122.1 and 120.4 K respectively, which are higher than those of 122.6, 116.0 and 113.4 K for the latter. The resistance of the new phase is much higher than that of the 2223 phase, which would be caused partly by impurity phases and partly by grain boundaries.

Magnetic susceptibility measurements for the new phase sample showed an onset temperature of the superconducting transition of 122.0 K, in agreement with the  $T_{c0}$  value measured resistively. The amplitude of the Meissner signal in the new phase sample was over half that in a well prepared  $\text{YBa}_2\text{Cu}_3\text{O}_{7-y}$  superconductor<sup>6</sup>, indicating bulk superconductivity from the new 1234 phase rather than from the 2223 or 2212 phases mixed in small amounts. The magnetization in the low-Ti samples was saturated above 10 Oe. The saturation field is lower than that of  $\text{YBa}_2\text{Cu}_3\text{O}_{7-y}$  by a factor of about 10. This implies very low  $H_{c1}$  and very high  $H_{c2}$  values, which would be smaller and larger respectively than those of  $\text{YBa}_2\text{Cu}_3\text{O}_{7-y}$  by a factor of  $\sim 10$ .

The new superconductor family of  $\text{TiBa}_2\text{Ca}_{n-1}\text{Cu}_n\text{O}_{2n+3}$  ( $n = 1-5$ ) has also been prepared, and the lattice constants follow

the  $c$ -axis rule  $c = 6.3 + 3.2n$  for the single Ti-O layer compound. They have  $T_c$  values of  $<17$  K for  $n = 1$ , 91 K for  $n = 2$ , 116 K for  $n = 3$ , 122 K for  $n = 4$  and  $<120$  K for  $n = 5$ . The highest  $T_c$  value was obtained for the  $n = 4$  sample, which has four Cu-O layers in a unit cell. This suggests that for high  $T_c$  the most suitable average valence of the copper ion is around +2.25, or that the most suitable  $\text{Cu}^{3+}$  ion concentration is  $\sim 25\%$ .

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## Internal seiches and subaqueous landforms in lacustrine cohesive sediments

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The introduction of sidescan sonar to surveying the floors of Scottish Lochs has permitted recognition of low-relief blister-like slides on the slopes of major basins. Here we report that these features occur discontinuously, but in regularly distributed zones, in the lochs examined, at the level of the summer thermocline. Internal seiches on the thermocline create circulation patterns dominated by periodically reversing horizontal currents in nodal positions, and vertical motion at antinodes. Although the currents are not strong enough to entrain the deposited flocculent cohesive sediments, we suggest that they exert sufficient shear stresses at nodal regions to cause breakdown of floc structures. This gives rise to 'shear thinning' of the surficial sediments and a consequent deepening of the weakened near-bed concentrated suspensions which, because they are situated on 8-18° sloping surfaces, begin to move downslope. As water escapes, shear strength is regained and the sediment ceases to move, leaving zones of blister-like slides centred on the nodal sections of the basin slopes. At the antinodes, no slides are developed.

The reservoirs and lochs of Scotland are sites of active sedimentation, mainly of coarse pebbly sands in shallow water and of fine cohesive silts and clays in deeper water. During the past 10 yr we have been engaged in examining the rates of sedimentation, the principal processes involved, and the nature of the end products<sup>1-5</sup>. Following the initial bathymetrical surveys of Murray and Pullar<sup>6</sup>, we have recharted many of the water bodies of the Grampian Highlands and the Midland Valley, using echosounders of various frequencies<sup>7</sup>. By introducing sidescanning sonars to the lakes and reservoirs, we have detected suites of subaqueous features<sup>8,9</sup> and confirmed many of the suggested interpretations from surveys of reservoirs before and after drawing down of the water levels<sup>10</sup>.

The most novel features<sup>8</sup> were the blister-like slides, described as occurring in isolation or in clusters, on the upper parts of the basin slopes. First reported from surveys in L. Earn, the slides have since been recognized in L. Tay and L. Lubnaig. When originally described it was estimated that their relief was 1-2 m, but subsequent diver investigation found that most reach no more than 0.25-0.5 m; however, the slides do not occur continuously along the upper slopes of the entire basin. Analysis

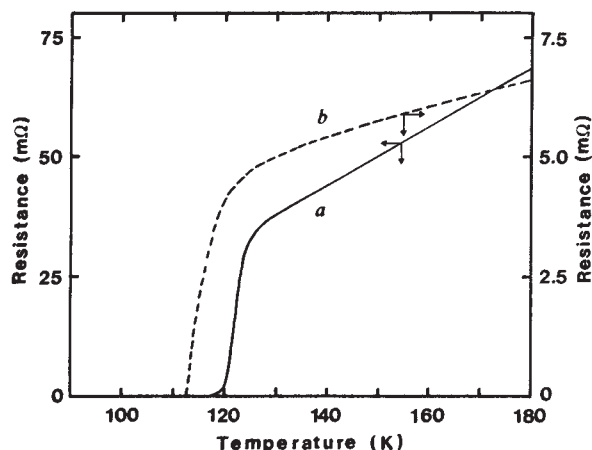
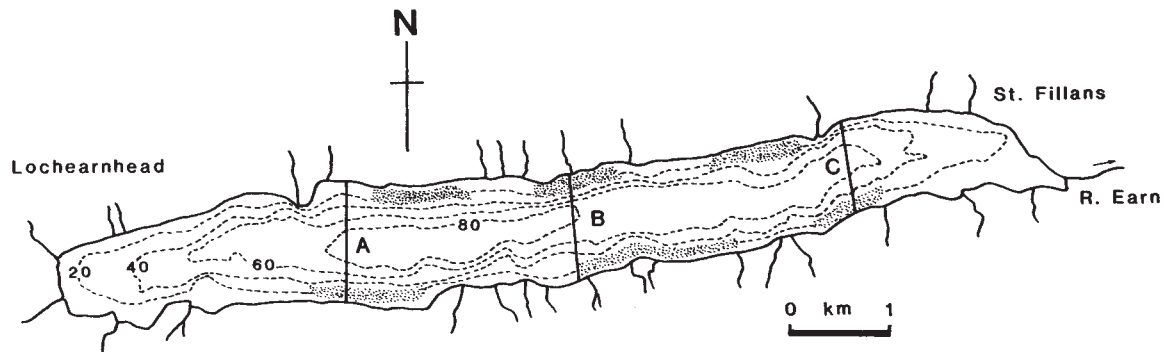
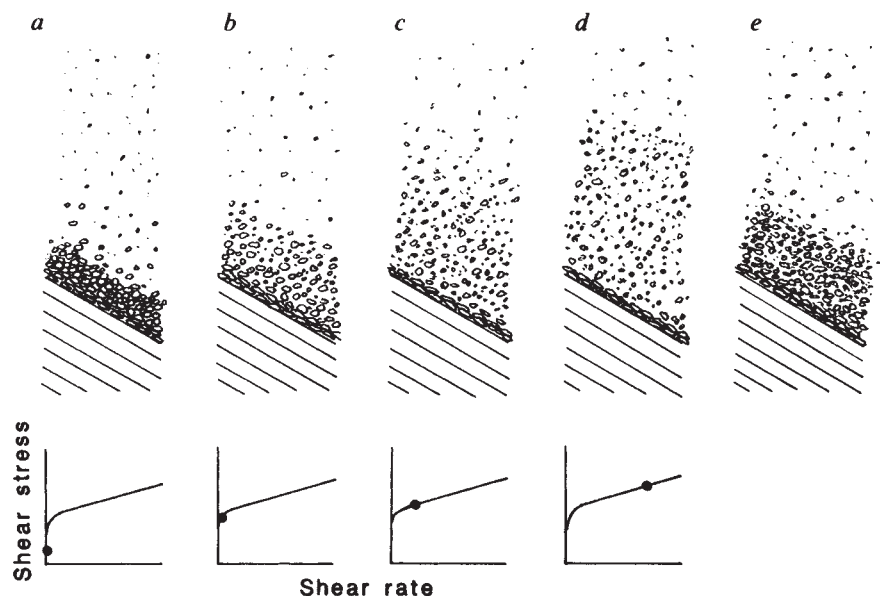


Fig. 2 Resistance versus temperature for the new  $\text{TiBa}_2\text{Ca}_3\text{Cu}_4\text{O}_{11}$  (1234) phase sample (a) and an intermediate sample with the (2223) phase (b).



**Fig. 1** Map of L. Earn ( $56^{\circ}23' \text{ N}$ ,  $4^{\circ}7' - 4^{\circ}17' \text{ W}$ ) showing the distribution of 'zones' of blister-like slides (dotted areas) around the basin slopes as recognized by sidescan sonar. The intensity of sliding decreases towards the ends of each zone. The lines A and C mark the calculated positions of the western and eastern seiche binodes and B the calculated position of the uninode<sup>13-15</sup>. Skeleton bathymetry shows the 20, 40, 60 and 80 m contours (after Al-Ansari and McManus<sup>1</sup>).

**Fig. 2** Definition diagram illustrating, qualitatively, the changes in bed structure and the shear-thinning rheogram for deforming lake floor muds. *a*, Early shear disrupts neither compressed nor undeformed flocs; *b*, threshold of shear separates upper flocs; *c*, shear breaks apart flocs and deepens deposited mud layer; *d*, continued shear and floc fracture deepens muds until downslope motion occurs; *e*, escape of water during motion, reformation of flocs and bed deposition. Increasing shear follows the progressive sequence from *a* to *d*. State *e* is reached as the sediment resettles after sliding.



of continuous sonographs from the entire lengths of the shores of L. Earn and L. Tay reveal zones in which the blister-like slides are absent, although the bed slopes ( $8-18^{\circ}$ ) and bed materials (flocculated cohesive organic rich silts) are identical to those where the slides are developed.

Their distribution and discontinuous nature suggest the mechanism of formation of these features. The observed depths of occurrence, most commonly 15–25 m, correspond to those at which the thermocline, separating warm waters of the surficial epilimnion from those of the colder, denser hypolimnion at depth, forms during the period June to late August<sup>3</sup>. The thermocline is least well developed when at shallow depth, in early summer, but deepens and strengthens progressively until by late August it forms a well-marked pycnocline with a steep gradient.

The physical behaviour of the water body of L. Earn received detailed study by Chrystal<sup>11,12</sup> and Chrystal and Wedderburn<sup>13</sup>, who examined principally the seiche phenomenon in the surface waters (barotropic seiche), demonstrating the positions of nodes of the uninodal and binodal seiches (Fig. 1) with amplitudes of a few centimetres. By contrast Wedderburn<sup>14</sup> and Wedderburn and Young<sup>15</sup> explored the activity of internal waves on the thermocline by using records of temperature-profile variations measured at hourly intervals over many days. They revealed uninodal and binodal seiche periods of  $\sim 15$  and 8 h respectively on several occasions, with measured internal (baroclinic) seiche amplitudes of up to 16 m, but commonly 3–10 m. The greatest

associated horizontal current speed measured was  $7 \text{ cm s}^{-1}$ . The internal seiche takes the form of a standing wave in which motion at the antinodes is vertical but motion at the nodes is effectively horizontal, and is associated with periodically reversing bottom currents.

Reversing to Mortimer<sup>16</sup> the nodes of the internal and surficial seiches are often geographically coincident, as demonstrated by measurement<sup>17</sup> and theoretical analysis<sup>18</sup> of water motions in L. Geneva. The work of Chrystal<sup>11,12</sup> and Wedderburn<sup>14,15</sup> has shown that the seiches of L. Earn are symmetrical, unlike the strongly asymmetrical internal seiches demonstrated for L. Ness by Thorpe<sup>19</sup> and his co-workers<sup>20</sup>. As a result the locations of sectors dominated by horizontal or vertical water motion are readily identified.

Three of the six 'zones' of blister-like slide occurrence are coincident with the nodal positions calculated by Chrystal and Wedderburn<sup>13</sup>, two on the south side of the loch and one on the north (Fig. 1). The other three 'zones' are displaced from the calculated nodal positions. However, the centres of these slide 'zones' are always within 1 km of the predicted nodes and the spacings between the groups of slides, along each side of the basin, are very similar in scale to those of the predicted nodal spacings. Hence we suggest that the formation of the zones of blister-like slides may be linked with internal seiches on the thermocline. If particles of sediment are to be carried along the bed, the shear stresses exerted upon them must exceed



the threshold required for motion to start in these cohesive sediments. But there are no detectable bedforms indicating along-slope transport, whereas the asymmetry of the blister-like slides indicates down-slope migration. Therefore current-induced sediment transport *per se* cannot be invoked.

In the nodal areas of the internal seiche the horizontal components of water motion are at their greatest. A speed of  $7 \text{ cm s}^{-1}$  measured at 20 m depth was determined in the central part of the loch during unexceptional conditions, associated with a seiche of 5 m height (2.5 m amplitude)<sup>15</sup>. More substantial seiches doubtless generate greater current speeds but it is unlikely that these exceed the critical threshold values required to permit entrainment of deposited cohesive sediments. Nevertheless we suggest that the shear stresses exerted by the internal seiche-associated reversing currents are capable of disturbing the internal structure of the deposited sediments.

The imperfect coincidence between the zones of blister-like slides and the calculated nodal positions may be attributed to a number of factors. The possibility of interfering lateral seiches can be almost discounted owing to the combination of high mountains to the north and south of the loch plus the very restricted fetch in these directions. Locally, however, the bathymetry of the basin has been shown<sup>1</sup> to be not as simple as indicated by the chart of Murray and Pullar<sup>6</sup>, used as the basis for Chrystal and Wedderburn's calculations<sup>13</sup>. Locally, previously unrecognized shelves and hollows at shallow depth may modify seiche-generated bottom currents. During flood discharges, variations in lateral tributary stream inputs may also exert some, albeit minor, influence on the nodal positions. Thus we suggest that the pattern of nodal positions is not as simple as predicted but detailed investigation would be necessary to identify the actual nodal positions.

The nature of the materials that have accumulated on the loch floor slopes is largely determined by settling from suspension. Although in the water column concentrations rarely exceed  $10 \text{ mg l}^{-1}$ , in the near-bed zone concentrations rise and aggregates are generated. Once concentrations exceed  $10 \text{ g l}^{-1}$ , inter-aggregate contacts permit a structural framework to develop, effectively creating a recognizable 'bed'<sup>21</sup>. As further material accumulates above, the aggregates deform as they undergo gravity-induced compaction. Their shear strength increases with time<sup>22</sup>, as does their resistance to potential erosion.

As currents pass over such materials, aggregates above the 'bed' may become displaced. As shear stresses increase, the consolidating bed experiences deformation during which aggregate structures break down under stress, and 'shear thinning' of the deposit occurs<sup>23</sup>. Similar shear thinning has been frequently reported in natural cohesive or flocculated suspensions subjected to low steady shear rates<sup>24</sup>. In the uppermost part of the bed the aggregates are broken apart to produce large numbers of smaller aggregates separated by water. This effectively causes an upward diffusion of sediment from the partly consolidated bed, producing a deeper layer of concentrated suspension (Fig. 2). The mass of material above the consolidated bed thus increases, without necessarily increasing the suspension concentration.

Bed material swelling due to inter-floc rearrangement observed by Mehta and Maa<sup>25</sup> in wave-tank experiments leads to a reduction of the effective shear strength of the bed. The layer of concentrated suspensions becomes thicker than that developed at any stage during deposition through settling.

We suggest that as the suspension layer deepens its centre of gravity rises and its density, viscosity and shear strength decrease so that, at sites with suitable bed gradient, downslope motion under the influence of gravity begins. As the sediment moves, water escapes progressively, the concentrations increase with resultant reaggregation, densities and shear strengths return to their former levels, and motion ceases (Fig. 2e). By this stage the blister-like structure has formed and becomes stabilized on

the slope side. The horizontal components of current-induced shear stresses are at their greatest in nodal zones and so the blister-like slides develop on these sectors of the lake slopes.

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## Episodic aseismic earthquake precursors

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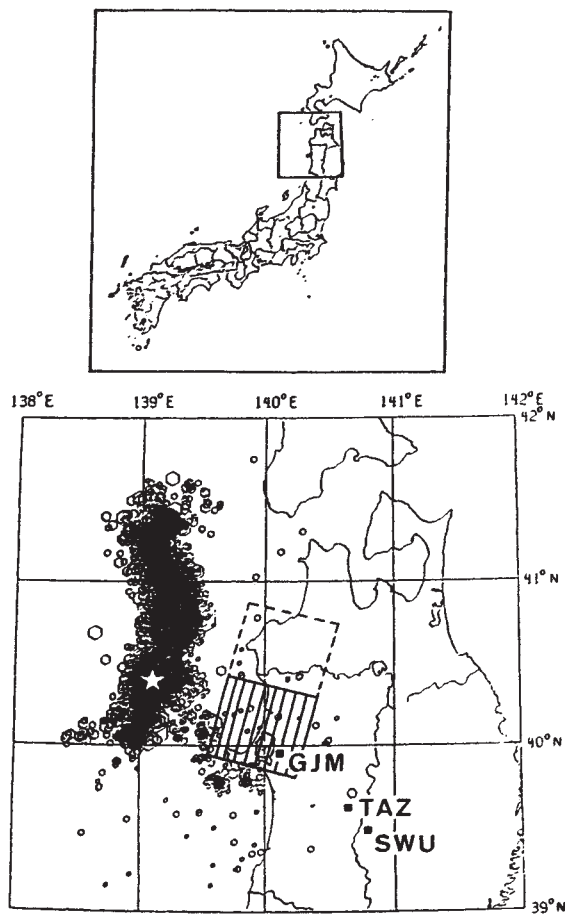
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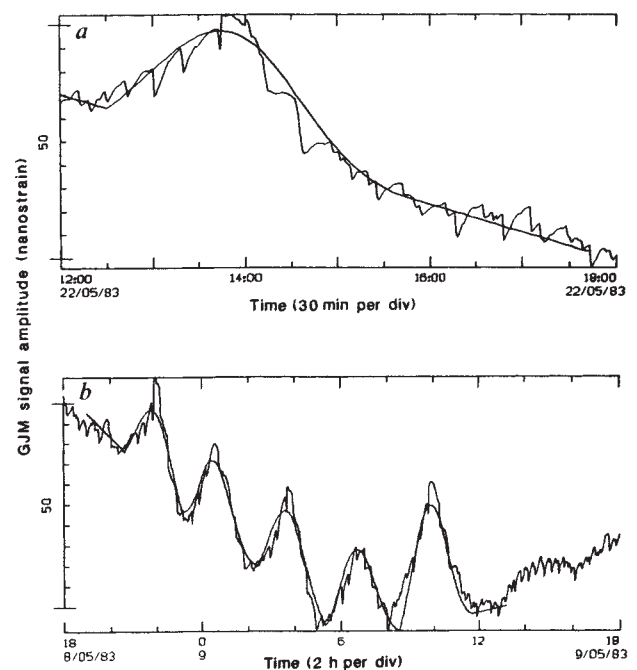
Shallow earthquakes are generally believed to be brittle fractures in a stressed medium with rupture velocity at a speed close to that for shear waves. We know, however, that the Earth allows failure over a wide range of timescales. Creep events occur on the San Andreas fault system, and for some earthquakes the high-speed rupture is accompanied by a slower process<sup>1–5</sup>. Here we report on episodic slow events which occurred before the Japan Sea earthquake of magnitude 7.7 on 26 May 1983. A Sacks–Evertson borehole strain meter<sup>6</sup> 90 km from the earthquake recorded about 100 aseismic strain events in a five-month period before and immediately following the earthquake; none has been detected after the large aftershocks. The observed signals are consistent with a precursory redistribution of stress through aseismic slip on a deep extension of the main-shock fault plane. Such episodic aseismic events may provide a mechanism for relatively rapid stress concentration before earthquakes.

Three borehole instruments were installed in Tohoku, northern Honshu in October 1982. Figure 1 shows the locations of these stations: GJM, TAZ, and SWU. In May 1983, the Japan Sea earthquake of magnitude 7.7 occurred, and from the aftershocks we see the extent of the fault plane (Fig. 1). These aftershocks, determined from the Tohoku seismic network, indicate that the fault plane dips at about 20 degrees to the east.

During the interval between initial operation of the instruments (December 1982) and the large earthquake, about 100



**Fig. 1** Tohoku area in northern Honshu. Japan Sea earthquake ( $m = 7.7$ ; 26 May 1983) epicentre is shown by an open star. Aftershocks (dark area) indicate the extent of the fault plane that dips at about  $20^\circ$  to the east. GJM, TAZ and SWU are the locations of 3 borehole strain meters. The hatched area indicates the plane of aseismic slip. The map location is shown above.



**Fig. 2** Examples of the aseismic strain events. Tides have been removed from the data, but no correction has been made for atmospheric pressure changes as pressure was not recorded at GJM during this time interval. Note that the time scales are different for the two plots. *a*, A single event (22 May) and the model-derived trace (smooth curve). The fault area used is 50 km by 65 km with a slip of 1.5 cm. Slope in the data is due to atmospheric pressure change (verified from the pressure recorded at nearby OGA); the model values have had a matching trend added. *b*, A suite of 5 events in succession (8–9 May). The superposed smooth trace results from the addition of time-delayed events together with matching slopes. The same source fault plane as in *a* is used for all events in *b*. Slip amplitudes for the 5 events in *b* are 1.25 cm, 1.5 cm, 1.25 cm, 1.25 cm and 1.8 cm.

unusual slow events were recorded by the GJM instrument. Examples are shown in Fig. 2. The events are similar in shape, have durations of about 3 hours and amplitudes 10 to 30 nanostrain. (GJM has a local repetitive high-frequency noise source; otherwise it exhibits stable behaviour and has good long-term characteristics.) Because variations in atmospheric pressure produce strain changes in the crust, we have verified that these signals are not due to such variations by examining the nearest available pressure record from the Oga peninsula (OGA), about 30 km distant from GJM. The shape of these events is such that they cannot be due to precipitation; nevertheless we verified that there is no correlation between the event times and times of precipitation.

At the other two stations, TAZ and SWU, we are unable to identify any signal that could be due to the same source. Any such signal amplitudes must be less than about 10% to 20% of those at GJM; our proposed model is consistent with these null observations.

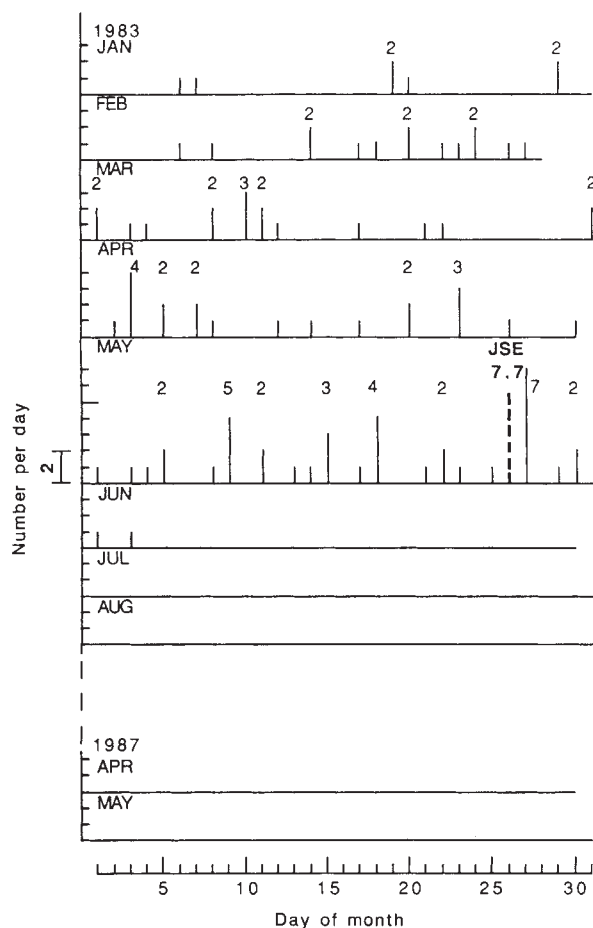
We have examined and then rejected the possibility that these events could be transient effects due to the installation procedure and that they ceased as a result of coseismic acceleration effects. More than 80 similar instruments have now been installed in various countries. In a few sites, high-frequency noise (similar to that at GJM) is generated very close to the instrument because of stressing by the expansive grout used. (With modified installation techniques, we have been able to eliminate this unwanted signal.) Those signals are consistently highly asymmetric, with sharp rises and slow recovery; they exhibit strong periodicity

and usually diminish in strength on a time scale of years. We have no other examples of the bay-like signals (Fig. 2) attributable to installation effects, and we have observed similarly shaped signals in Iceland and California which initiate years after installation and are correlated in space and time with tectonic activity. Since the Japan Sea earthquake, the high frequency noise signals at GJM have continued and there is no change in the coupling of the instrument to the Earth, as evidenced by the amplitude of tidal signals and the measured strain induced by atmospheric pressure changes. Additionally we note that for more than a week after the main shock there were more strain events with the same characteristics. All of our experience with borehole strain data indicates that these signals are not due to installation transients—in fact, even before the occurrence of the Japan Sea earthquake, they were noted as being unusual and perhaps significant.

The occurrence history of these events (Fig. 3) is compelling evidence that they are closely related to the Japan Sea earthquake. After the main shock, some more strain events occur (including several in a multiple sequence two days later), but there are none after the large aftershocks (largest magnitude 6.7). No similar events have since been recorded at GJM.

Considerations of physical plausibility allow exclusion of mechanisms such as an oscillating pressure source close to GJM, or nearby changes in pore pressure (the observed signal shape would not be symmetrical). We have already excluded the possibility of atmospheric sources, and so the remaining mechanism is slow propagation of slip on some surface. We make the reasonable assumption that, because of the chronology of the events, the source geometry is simply related to that of the

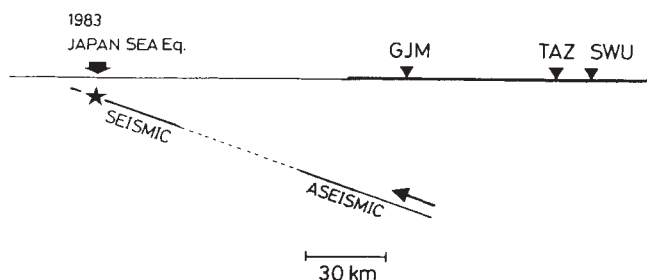




**Fig. 3** Time history of event occurrence in number per day. The instruments began operation at the beginning of December 1982. Some events may have been recorded before January 1983, but recognition is more difficult in the early data. Numbers indicate multiple events. No similar events have occurred since the large aftershocks.

earthquake; thus we consider only extensions of the earthquake fault plane. We proceed now to test that assumption.

Only a down-dip extension of the fault plane provides consistency with the data. Aseismic slip on a similar surface has previously been proposed<sup>7</sup> to explain preseismic uplift on the west coast of Tohoku. Figure 4 shows a vertical section of our model; in Fig. 1 the proposed plane of aseismic slip is shown in map projection. The aseismic slip on this deep extension is thrust motion which propagates upward unilaterally. Figure 3 shows the calculated time-variation of dilatation for such a model superposed on the data. We use the formulation by Okada<sup>8</sup> for these calculations. The fault parameters are a length of 50 km and a width down-dip of 65 km; the thrust slip, constant over the fault plane, varies from 1.25 cm to 1.8 cm for the examples shown. The rupture velocity is held constant at 20 km per h ( $5.5 \text{ m s}^{-1}$ ). The signals calculated for GJM have the correct polarity, and amplitudes and shapes comparable with those observed; the signals calculated for TAZ and SWU are below the threshold of detection. Note that simple ideas based on an inverse cube relation and concerning the relative signal amplitudes to be expected at these sites are misleading, because the sites are all relatively close to an extended source. Fault lengths of 25 km, 50 km and 100 km all produce essentially the same signals. Because of the similarity of shape throughout the event sequence, the down-dip locations of the initiation and cessation of rupture do not vary significantly. All the recorded examples have similar shapes and so the same basic model produces a good fit to all events.



**Fig. 4** Vertical section (no vertical exaggeration) indicating region of aseismic slip. Thrust type slip propagates upwards at a velocity of  $20 \text{ km h}^{-1}$ .

The model was constrained by using the main shock fault geometry and the GJM borehole strain data only, but we get consistency with the lack of observations at the other 2 sites. Also there is persuasive consistency with other observations of crustal deformation. Calculated uplift, integrated over all events, is consistent (at the factor of 2 level) with tide gauge data and levelling surveys<sup>7</sup>. For the accumulated tilt at OGA and GJM, the calculated magnitude agrees within a factor of 2 with the observed tilts of a few microradians; the directions agree within 45 degrees. Because of the difficulties inherent in surface measurements and our limited control on the model parameters, these agreements are satisfactory. All available tests of our assumption that the signal source is simply related to the main shock are positive.

Other evidence supports this interpretation. Similar signals (study in progress) have also been recorded in southern Iceland, close to the southern transform fault, where there is an enhanced potential for a moderate earthquake. A comparable process may also be occurring near Parkfield, California. Rock mechanics experiments<sup>9</sup> support the concept of slow precursory slip.

The recorded signals have good signal-to-noise ratio, occur frequently and have a strong temporal association with the Japan Sea earthquake. We interpret them as clear evidence for episodic precursory changes, presumably involving stress redistribution. The model we present here is certainly not unique, but we assume a reasonable connection with the main shock. The model provides a very good fit to the data and is consistent with other measurements of crustal deformation. The deformation produced by the proposed source results in higher stress concentration in the region of the eventual large shock; perhaps as much as 20% of the seismic stress release was added in the 6 months immediately preceding the main shock. We suggest that aseismic precursory events provide a significant mechanism for stress redistribution before earthquakes. If this scenario is correct, then such a mechanism may provide an explanation for some of the examples of anomalous behaviour preceding the earthquakes catalogued by Rikitake<sup>10,11</sup>.

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# Copper inclusions in sheet silicates from porphyry Cu deposits

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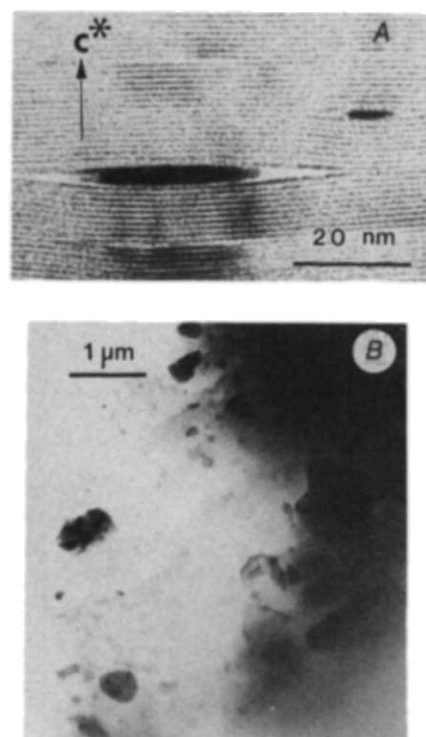
Economic geologists have studied the Cu contents of biotite for at least a quarter of a century as potential guides for exploration and as clues to the genesis of porphyry copper deposits<sup>1-12</sup>. These earlier studies used bulk analytical techniques, and a common, although cautious, assumption was that Cu substituted for Fe in the octahedral sites of the biotite. Here we report data from high-resolution and X-ray analytical transmission electron microscopy which demonstrate that anomalous Cu in biotite is present primarily as submicrometre inclusions of pure native Cu in the interlayer regions of the mica. The inclusions occur with greatest density and size in mixed-layer sheet silicate domains which form by alteration of biotite and chlorite. Less abundant and smaller Cu inclusions are associated with biotites that show subtle alteration only on the high-resolution transmission electron microscopy scale. The association of Cu inclusions with alteration domains, coupled with mass-balance considerations, suggests that these sheet silicates act as Cu sinks, and not as sources for primary mineralization.

Banks<sup>13,14</sup> used specimens from intrusive rocks near the porphyry copper deposits at Ray and Esperanza (Arizona) to study the geochemistry of Cu in biotite: he showed that Cu is concentrated in altered regions of biotites using the petrographic microscope, scanning electron microscopy and electron microprobe analysis. Cu levels were found to be highest in narrow swaths of an unidentified phase (up to a startling 13 wt% when calculated as CuO), chlorite contains up to several thousand p.p.m. Cu, whereas biotite usually contains less than 90 p.p.m. Cu. The Cu content is not correlated with sulphur, which suggested that it was incorporated in the sheet silicates, rather than being present as submicroscopic inclusions of a Cu-bearing sulphide. Rehrig and McKinney<sup>15</sup> have also described subtle alteration of biotite from several porphyry Cu deposits and found that the alteration is correlated with high Cu content.

During extensive electron and ion microprobe analyses, Hendry *et al.*<sup>16,17</sup> noted alteration textures similar to those reported by Banks, and similar distributions of Cu in biotite and its alteration products both at the Koloula complex (Solomon Islands) and at some North American porphyry Cu deposits. Yet in some samples Hendry *et al.* report up to 5,000 p.p.m. Cu in primary igneous biotites, and up to 2 wt% Cu in chloritized domains of hydrothermal biotites.

There are major unresolved mineralogical and geochemical problems associated with the sheet silicates from porphyry Cu deposits. The source and structural state of their anomalous Cu have not been identified; it is not known from available data whether the unidentified high-Cu sheet silicate is a mica, vermiculite, chlorite, smectite, hydrobiotite, mixed-layer clay, or some other material; and details of how the sheet silicate alteration reactions take place, and how the reactions relate to the behaviour of Cu in an evolving porphyry Cu deposit are lacking.

Here we present initial transmission electron microscope (TEM) results on the distribution and occurrence of anomalous Cu in chlorites and in igneous and hydrothermal biotites from the Chikora tonalite porphyry, the Koloula igneous complex and from the porphyry (Oxhide) phase of the Schultze Granite, Globe Miami District, Arizona. Though the scales of observed textures vary, the results from both the Koloula and Globe Miami materials are similar, suggesting that our observations could have some generality. All the figures accompanying the following discussion derive from Koloula material.



**Fig. 1** TEM images of biotite containing Cu inclusions. *A*, High-resolution TEM image viewed perpendicular to  $c^*$ . *B*, Conventional TEM image viewed parallel to  $c^*$ .

The most important result of our study is the identification of the phase that carries the anomalous Cu in biotite, a problem that has long eluded economic geologists. In all the specimens studied, anomalous Cu occurs primarily as submicroscopic inclusions of native Cu metal. This is consistent with experimentally determined and calculated fluid/crystal Cu distribution coefficients<sup>18</sup>. High-resolution and conventional TEM images show that the Cu inclusions range from 2 to 100 nm in width and are up to ~1,000 nm in diameter, being generally flattened parallel to the layers of the enclosing sheet silicate (Fig. 1*A*). The inclusions lie in the interlayer regions and typically the sheet silicate is deformed around the inclusions, as in the case of inclusions of reaction products in biotites imaged by Veblen and Ferry<sup>19</sup>. Images obtained parallel to  $c^*$  of the sheet silicates show that the inclusions are discoid and can be twinned or intergrown in a complex fashion (Fig. 1*B*).

X-ray analytical TEM analyses of the larger inclusions (>20 nm) provide evidence for no elements other than Cu, but elements with an atomic number of less than 11 are not detected by this method. But selected-area electron diffraction patterns from all but one inclusion studied so far are consistent with Cu metal, and not with other Cu minerals. The X-ray analytical TEM and selected-area electron diffraction results taken together thus indicate that the Cu inclusions are primarily in the form of pure native Cu.

It is important to note that the Cu inclusions cannot be resolved by conventional optical methods or using the electron or ion probes. The fact that the inclusions appear even in subtly altered biotite (see below) should caution the interpretation of all Cu analyses of sheet silicates.

The results of our TEM study are consistent with the conclusion of Banks<sup>13</sup> that Cu is concentrated in the alteration products of igneous and hydrothermal biotite. Banks found less than 90 p.p.m. Cu in unaltered biotite. Our X-ray analytical TEM analyses of unaltered biotite indicate the presence of Cu



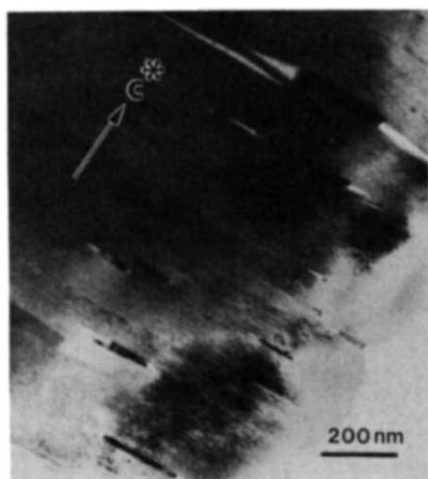


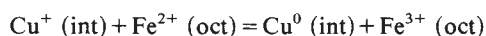
Fig. 2 Conventional TEM image illustrating the density of Cu inclusions in hydrobiotite.

in amounts below the detection limit (0.2–0.3 wt%), whereas altered domains contain up to 10 wt% Cu. The Cu inclusions and therefore the high Cu in chemical analyses are associated primarily with mixed-layer sheet silicate alteration products of biotite (Fig. 2). As these are chemically and crystallographically similar to hydrobiotite, as defined by Brindley *et al.*<sup>20</sup>, we shall refer to this alteration product as hydrobiotite. More limited numbers of inclusions also occur in biotite. These inclusions on average are smaller than those in hydrobiotite and generally occur in the proximity of hydrobiotite, namely near sites where the biotite has been replaced by this alteration product. High-resolution TEM images (see Fig. 3, for example) indicate that the copper inclusions in biotite typically lie along, or close to, interlayers that are slightly expanded or have lighter contrast than normal. Thus, the inclusions in biotite could be associated with isolated interlayers that have been vermiculitized.

It is interesting that well crystallized chlorite has not been found to contain the Cu inclusions. Like biotite, chlorite in these occurrences commonly contains narrow, submicroscopic lamellae of Cu-bearing hydrobiotite intergrown on (001) (Fig. 4). Variable volumes of such lamellae could account for the higher Cu contents previously reported for chlorite relative to biotite, and for the irregular distribution of Cu in chlorite (for example, see ref. 13).

The above observations suggest that anomalous Cu in the sheet silicates we have studied is not inherited from the parent biotite, but rather is introduced early in the hydrobiotite-producing alteration event.

Although our observations show that Cu-enrichment involves precipitation of native Cu, they do not define the precise reactions involved in this process. But the fact that the inclusions always lie in the sheet silicate interlayers and deform the hydrobiotite or biotite suggests that Cu is introduced, possibly as  $\text{Cu}^+$  ions, by penetration of a fluid along the interlayers. The Cu ions could exchange with  $\text{K}^+$ , with subsequent reduction of  $\text{Cu}^+$  to  $\text{Cu}^0$  by octahedral  $\text{Fe}^{2+}$  in the biotite:



The link between Cu precipitation and formation of hydrobiotite suggests that the two processes could occur simultaneously by a more complex reaction. Further analytical work at the X-ray analytical TEM scale will allow us to detail the mass balances for reaction of biotite to the various hydrobiotite compositions. This could lead in turn to a better understanding of the chemistry of Cu deposition in the hydrobiotite.

This study has produced a number of new observations relevant to the geochemistry of Cu in porphyry Cu deposits. An

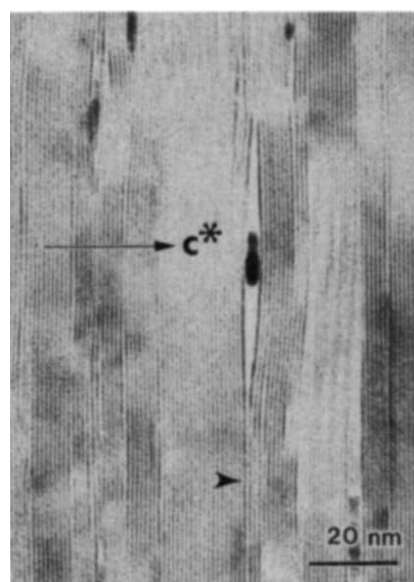


Fig. 3 High-resolution TEM image of Cu inclusions in biotite. Note the association of the inclusions with expanded interlayers. One expanded interlayer is arrowed.

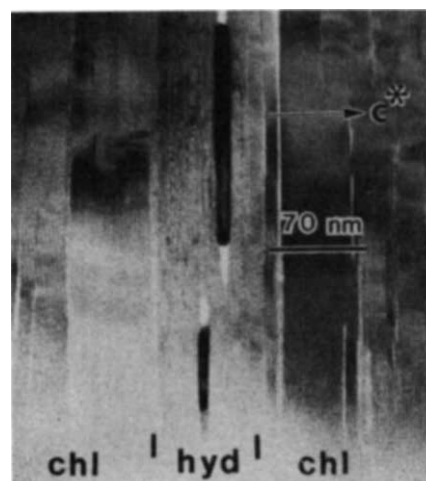


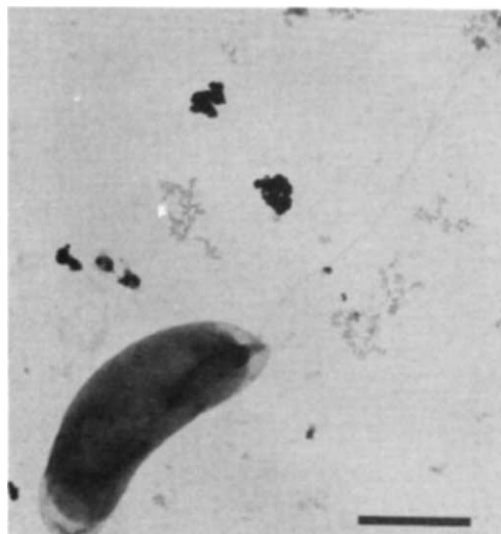
Fig. 4 High-resolution TEM image of intergrown chlorite (chl) and hydrobiotite (hyd). The Cu inclusions are restricted to the hydrobiotite lamella.

important implication of our interpretations is that biotites unusually enriched in Cu are not necessary for the formation of porphyry Cu deposits. A key question is whether the hydrobiotite and Cu inclusions that we have observed were formed in hydrothermal or weathering environments. Argillic alteration and associated hydromicas can occur during either hypogene or supergene alteration<sup>21</sup>. The rocks at Koloula, however, have seen insignificant supergene enrichment<sup>22</sup>. Given the appreciable lack of sulphides in our specimens, which would compete for Cu, a hydrothermal origin might reasonably explain our observations. But hypogene fluids that have extremely low  $f_{\text{S}_2}$  would be very unusual for porphyry Cu environments. Moreover, some evidence suggests that Cu enrichment in biotite may be restricted to oxidized rock above the water table<sup>15</sup>. It is therefore possible that we are documenting extremely subtle supergene alteration at Koloula. Further observations from different deposits are required to determine the environment and geochemistry of these events more rigorously.

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**Fig. 1** Electron micrograph of a negatively-stained vibroid cell of strain MV-1. Note the single polar flagellum and the chains of intracellular electron-opaque magnetosomes. The bacterium was stained with 2% phosphotungstic acid. Bar, 0.5  $\mu$ m.

## Anaerobic magnetite production by a marine, magnetotactic bacterium

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Bacterial production of magnetite represents a significant contribution to the natural remanent magnetism of deep-sea and other sediments<sup>1-5</sup>. Because cells of the freshwater magnetotactic bacterium *Aquaspirillum magnetotacticum* require molecular oxygen for growth and magnetite synthesis<sup>6</sup>, production of magnetite by magnetotactic bacteria has been considered to occur only in surficial aerobic sediments<sup>7</sup>. Moreover, it has been suggested that deposits of single-domain magnetite crystals are palaeoxygen indicators presumably having been formed under predominantly microaerobic conditions<sup>5-8</sup>. In contrast, some nonmagnetotactic, dissimilatory iron-reducing bacteria, such as the recently described strain GS-15 by Lovley *et al.*<sup>7</sup>, synthesize extracellular magnetite from hydrous ferric oxide under anaerobic conditions. We now report the first isolation and axenic culture of a marine, magnetotactic bacterium, designated MV-1, that can synthesize intracellular, single-domain magnetite crystals under strictly anaerobic conditions. We conclude that magnetotactic bacteria do not necessarily require molecular oxygen for magnetite synthesis and suggest that they, as well as dissimilatory iron-reducing bacteria, can contribute to the natural remanent magnetism of even long-term anaerobic sediments.

Strain MV-1 was isolated from sulphide-rich sediment of an estuarine salt marsh near Boston, Massachusetts. Water samples from the site contained 23 p.p.t. salt. Cells were small (0.2-0.4 by 1-3  $\mu$ m) with vibroid to helicoid morphology and were motile by means of a single, polar flagellum (Fig. 1). Based upon morphology, guanine-plus-cytosine (G+C) content of the DNA

(53.5 mol %), and other characteristics, MV-1 does not appear to be closely related to *A. magnetotacticum*, presently the only other magnetotactic bacterium isolated in pure culture. Cells orientated and migrated along magnetic-field lines. Like bipolarly-flagellated magnetotactic spirilla<sup>9,10</sup>, an individual cell could migrate in either direction along the magnetic field lines and spontaneously reverse direction without turning around.

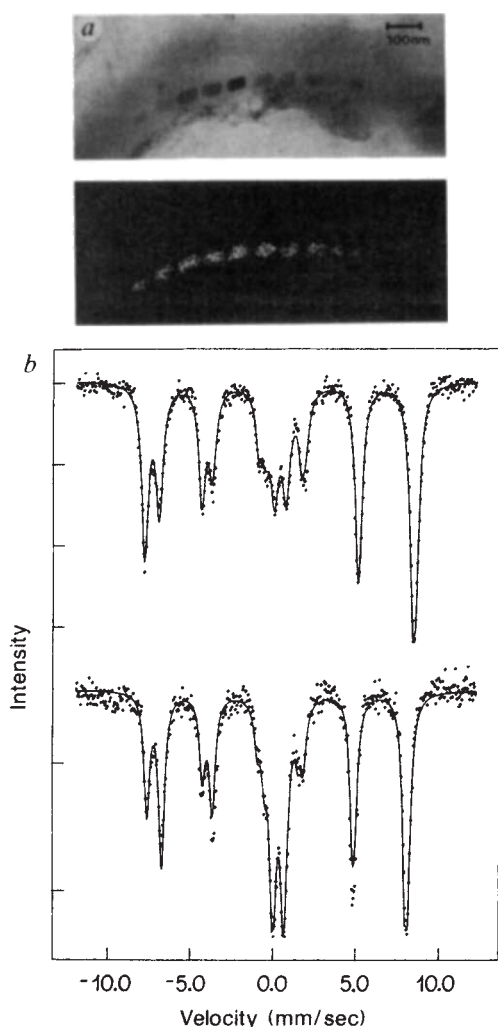
Cells of MV-1 oxidize organic acids and possibly certain amino acids anaerobically using nitrous oxide as terminal electron acceptor. Nitrous oxide is reduced to dinitrogen. To ensure the presence of anaerobic conditions, growth medium was boiled, cooled and dispensed under nitrogen gas passed over heated copper filings to remove oxygen. The medium was strongly redox buffered with 0.4 g l<sup>-1</sup> cysteine · HCl and contained resazurin, a redox indicator that is colourless when reduced. Dinitrogen in culture bottles was replaced with nitrous oxide that was passed through a solution of alkaline pyrogallol to remove traces of contaminating oxygen<sup>11</sup>. All medium used in this study was colourless prior to and just after inoculation. Oxygen was not detected in the headspace of culture bottles by gas chromatography.

Cells grown under strictly anaerobic conditions in pre-reduced medium with nitrous oxide and 25  $\mu$ M ferric quinate, contained a chain of approximately ten iron-rich magnetosomes that longitudinally traversed the cell (Fig. 2a). Iron constituted about 1.6% of the dry weight of the cells, and was highly localized in the magnetosomes. Magnetite, Fe<sub>3</sub>O<sub>4</sub>, was identified as the principal component of the magnetosomes by Mössbauer spectroscopy of freeze-dried cells and extracted magnetosomes (Fig. 2b). Hydrous ferric oxide, presumably a precursor to magnetite precipitation<sup>12</sup>, was also present. Cells are also able to grow microaerobically in semi-solid oxygen gradient cultures. Under these conditions, cells also behaved magnetotactically and contained an average number of eight magnetosomes per cell.

Individual magnetite particles were parallelepipeds with approximate dimensions 40 × 40 × 60 nm. This volume is within the single-magnetic-domain size range for magnetite<sup>13</sup>. An average permanent magnetic dipole moment per cell of  $M = 2.1 \times 10^{-13}$  e.m.u. was determined by measuring the total magnetic moment of a suspension of a measured number of cells with a SQUID magnetometer at room temperature in applied fields of 10-100 Oe. This is sufficient for the magnetotactic response<sup>14</sup>. Bulk magnetic measurements on freeze-dried cells gave a saturation magnetization corresponding to 1.5%

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**Fig. 2** *a*, Transmission electron micrograph of a portion of a cell of MV-1 showing chain of 10 magnetosomes (top) and iron X-ray map of the same region (bottom) showing localization of iron in the cell. Bar, 100 nm. *b*, Mössbauer spectra of extracted magnetosomes (top) and freeze-dried cells (bottom). The spectra correspond to  $\text{Fe}_3\text{O}_4$  with some surface oxidation of extracted magnetosomes. The extra quadrupole doublet close to zero velocity in each spectrum is presumably due to a hydrous ferric oxide precursor to  $\text{Fe}_3\text{O}_4$  precipitation. The solid line is a theoretical least-squares fit to the data.

magnetite per dry cell weight. The coercive force was 300 gauss and the remanent-to-saturation magnetization ratio approached 0.5. These values are comparable with those for *A. magnetotacticum*<sup>15</sup>.

Karlin *et al.*<sup>16</sup> showed that authigenic magnetite formation in suboxic marine sediments is confined between levels of nitrate reduction and iron reduction. Magnetotactic bacteria have not been considered the probable source of this magnetite, based on the molecular oxygen requirement of *A. magnetotacticum* for magnetite production. However, our results show that some marine magnetotactic bacteria can grow and synthesize single-magnetic domain particles of magnetite in the absence of molecular oxygen.

The occurrence of nitrous oxide is common in marine sediments<sup>17-19</sup>, presumably as a product of bacterial denitrification when oxygen is absent or in very low concentrations<sup>20,21</sup>. For this reason, organisms like MV-1 could be common in marine sediments in the layers reported by Karlin *et al.* as being the site of authigenic magnetite production. Dissimilatory iron-reducing bacteria could also contribute to the remanent magnetization of those sediments. However, the dissimilatory iron-

reducing bacteria produce magnetite particles with a wide size distribution that is strongly peaked in the superparamagnetic size range, and therefore below the size required for stable, single-magnetic domains (D. R. Lovley and R. B. Frankel, unpublished data). Thus magnetotactic bacteria, with their uniform, stable, single-magnetic-domain-size particles could be the principal contributors to the natural remanence even in suboxic marine sediments.

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## Ribosomal RNA sequence shows *Pneumocystis carinii* to be a member of the Fungi

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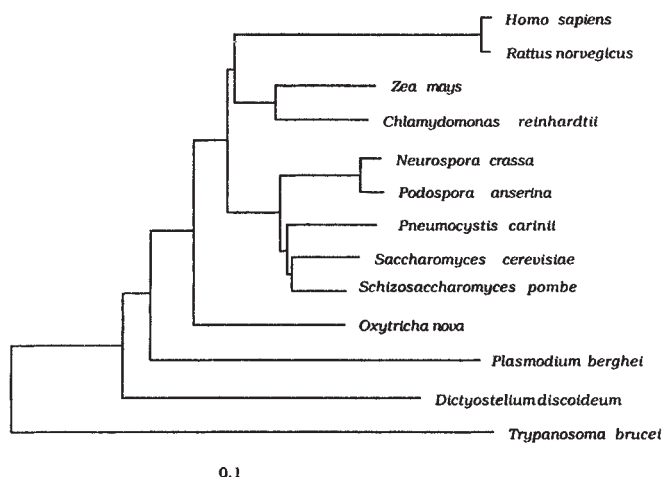
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*Pneumocystis carinii* pneumonia is the most common opportunistic infection in AIDS<sup>1</sup>, and accounts for significant morbidity and mortality in these and other immunocompromised patients<sup>2</sup>. *P. carinii* is a eukaryotic microorganism of uncertain taxonomy that can infect numerous mammalian hosts. Developing from a small, unicellular 'trophozoite' into a 'cyst' containing eight 'sporozoites', its life cycle superficially resembles those seen both in the Protozoa

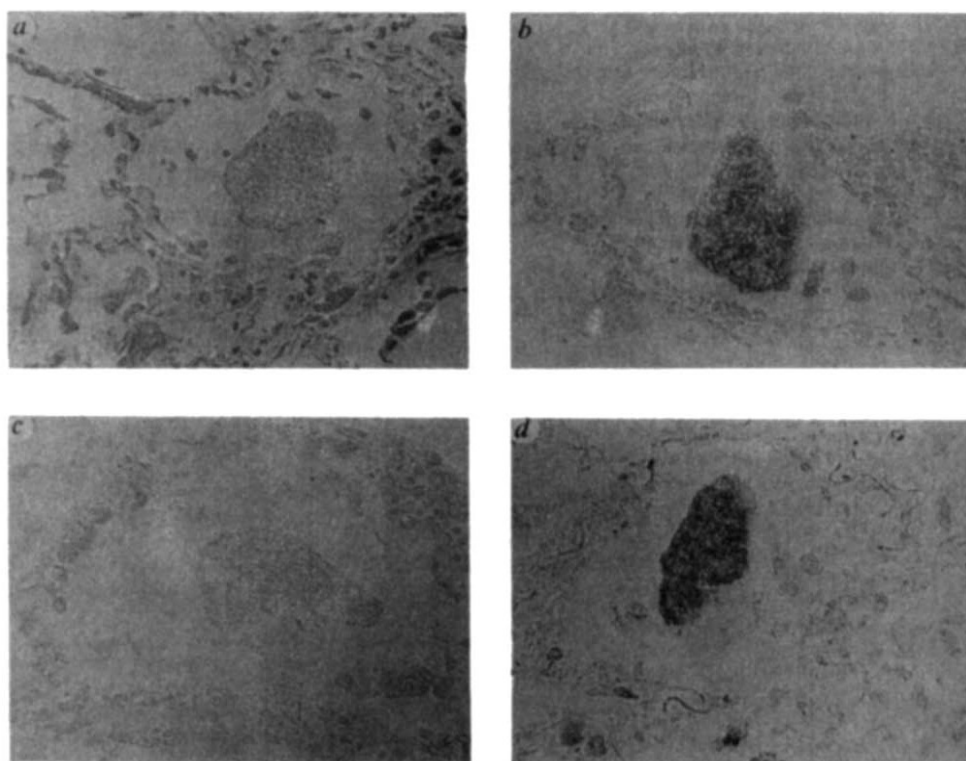
Structural similarities between the putative *Pneumocystis* 16S-like rRNA sequence and those of *Rattus norvegicus*, *Zea mays*,



**Fig. 2** Phylogenetic tree inferred from 16S-like- rRNA sequence similarities. Structural similarity values between the 16S-like rRNAs of *H. sapiens*<sup>27</sup>, *R. norvegicus*<sup>28</sup>, *Z. mays*<sup>29</sup>, *C. reinhardtii*<sup>13</sup>, *N. crassa*<sup>30</sup>, *P. anserina* (M.L.S., unpublished data), *P. carinii*, *S. cerevisiae*<sup>31</sup>, *Schiz. pombe* (M.L.S., unpublished data), *O. nova*<sup>12</sup>, *P. berghei*<sup>32</sup>, *D. discoideum*<sup>33</sup> and *T. brucei*<sup>11</sup> were converted into an unrooted phylogenetic tree by the distance matrix methods<sup>10</sup> as previously described<sup>12</sup>. The evolutionary distance between nodes of the tree is represented in the horizontal component of their separation in the figure.



**Fig. 3** Confirmation of the identity of the *Pneumocystis* sequence by *in situ* hybridization to *Pneumocystis*-infected human lung sections. *a*, Haematoxylin-eosin stain. *b*, *In situ* hybridization with biotinylated *Pneumocystis*-specific oligonucleotides. *c*, *In situ* hybridization with biotinylated *E. histolytica*-specific oligonucleotide. *d*, Immuno-staining with anti-*Pneumocystis* monoclonal antibody (7D7, ref. 16). A control monoclonal antibody to a neuron-specific glycoprotein<sup>34</sup> showed no reactivity (not shown).



**Methods.** *In situ* hybridization: Paraffin sections collected on polylysine coated slides were dewaxed in xylene, washed in absolute ethanol, allowed to air-dry, and rehydrated in phosphate-buffered saline for 10 min at room temperature. A hybridization mixture composed of 6×SSC, 5×Denhardt's, 10% dextran sulphate, and a mixture of the three biotinylated oligonucleotides<sup>14</sup> shown in Fig. 2 was applied to the section and covered with a glass coverslip. The slide was heated to 95 °C for 1 min and allowed to cool to room temperature. After 10 min at room temperature, the coverslip was removed and the slide washed in 6×SSC at 37 °C for 10 min. The slide was incubated with 1 µg ml<sup>-1</sup> streptavidin-alkaline phosphatase conjugate (BRL) in 0.1 M Tris-HCl (pH 7.5), 0.1 M NaCl, 2 mM MgCl<sub>2</sub>, 0.05% Triton X-100 for 10 min, washed twice in the same buffer, and developed with 330 µg ml<sup>-1</sup> nitroblue tetrazolium and 160 µg ml<sup>-1</sup> 5-bromo-4-chloro-3-indoyl phosphate for 30 min at 37 °C. The slides were washed in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and mounted in aqueous mounting medium. For immunostaining sections were dewaxed and rehydrated as above, then incubated with monoclonal antibodies in Tris-buffered saline (50 mM Tris-HCl pH 8.0, 150 mM NaCl) plus 0.05% Tween-20 (TBST) overnight at 4 °C. The sections were washed for 30 min in TBST and incubated with 1 µg ml<sup>-1</sup> biotinylated goat anti-mouse IgG (Boehringer) for 30 minutes at room temperature. After washing in TBST, the presence of the biotinylated second antibody was detected with streptavidin-alkaline phosphatase as described above.

*Sacch. cerevisiae*, *Neurospora crassa*, *Dictyostelium discoideum* and *Trypanosoma brucei* were compared<sup>12</sup> and are given in the upper right half of Table 1. Structural distances (average number of base changes per position) are given in the lower left half. Similarities between *Pneumocystis* and *Sacch. cerevisiae* (0.917) or *Pneumocystis* and *N. crassa* (0.904) are comparable to the similarity between *Sacch. cerevisiae* and *N. crassa* (0.913). These values are consistent with a close evolutionary linkage between *Pneumocystis* and the Fungi. The placement of *Pneumocystis* within a phylogenetic tree is shown in Fig. 2. This distance matrix tree<sup>10</sup> was constructed by converting similarity values to structural distances<sup>12</sup> and includes the organisms represented in Table 1 as well as *Homo sapiens*, *Chlamydomonas reinhardtii*,

*Podospora anserina*, *Schizosaccharomyces pombe*, *Oxytricha nova* and *Plasmodium berghei*. In this phylogeny and others<sup>13</sup>, protozoans are represented by several distinct lineages. In contrast, the Fungi form a close phylogenetic grouping which includes *Pneumocystis*. Thus, a close evolutionary linkage between *Pneumocystis* and protozoan groups can be ruled out.

To unequivocally establish that the sequence did represent *Pneumocystis* rRNA, oligonucleotides corresponding to the regions with least sequence identity with known 16S-like rRNAs (boxed in Fig. 1) were synthesized and used for *in situ* hybridization. Dot-blot analysis demonstrated that the oligonucleotides hybridized to RNA from several preparations of *Pneumocystis* trophozoites and failed to hybridize to uninfected rat lung RNA

**Table 1** Structural similarity and distance data between 16S-like rRNA sequences

| Organism                 | R.n.  | Z.m.  | S.c.  | N.c.  | P.c.  | D.d.  | T.b.  |
|--------------------------|-------|-------|-------|-------|-------|-------|-------|
| <i>R. norvegicus</i>     |       | 0.828 | 0.824 | 0.814 | 0.818 | 0.739 | 0.661 |
| <i>Z. mays</i>           | 0.195 |       | 0.862 | 0.859 | 0.862 | 0.773 | 0.673 |
| <i>Sacch. cerevisiae</i> | 0.200 | 0.152 |       | 0.913 | 0.917 | 0.773 | 0.673 |
| <i>N. crassa</i>         | 0.214 | 0.157 | 0.093 |       | 0.904 | 0.756 | 0.673 |
| <i>P. carinii</i>        | 0.208 | 0.153 | 0.088 | 0.103 |       | 0.767 | 0.667 |
| <i>D. discoideum</i>     | 0.320 | 0.270 | 0.270 | 0.296 | 0.279 |       | 0.662 |
| <i>T. brucei</i>         | 0.450 | 0.429 | 0.433 | 0.430 | 0.440 | 0.450 |       |

The upper right half of the table gives structural similarity values<sup>12</sup> for regions that can be unambiguously aligned in all of the considered 16S-like rRNA sequences. A total of 1,530 positions were considered. The structural distances (average number of base changes per sequence position) are shown in the lower left half of this table. References for all sequences are given in Fig. 2 legend.

or to RNA from a variety of bacterial and fungal pathogens (data not shown). Biotin-dUTP was added to the oligonucleotides using terminal transferase<sup>14</sup>. Such modification has been shown to have no effect on hybridization characteristics<sup>15</sup> and retention of specificity was confirmed by dot-blot analysis (data not shown). The biotinylated oligonucleotides were then used to probe tissue sections of *Pneumocystis*-infected human lung. A haematoxylin-eosin stain reveals the masses of *Pneumocystis* trophozoites and cysts filling the alveolar lumen (Fig. 3a). Hybridization *in situ* with the biotinylated oligonucleotides, followed by colorimetric detection, shows the hybridization to be confined to the alveolar lumen (Fig. 3b). A biotinylated oligonucleotide specific for *E. histolytica* rRNA did not hybridize (Fig. 3c). The organisms were confirmed to be *Pneumocystis* by immuno-staining with an anti-*Pneumocystis* monoclonal antibody (Fig. 3d; ref. 16). A control monoclonal did not react (data not shown). Co-localization of antigens and hybridization of rRNA-specific oligonucleotides verified that the sequence was from *Pneumocystis*. Based upon antigenic differences, it has been proposed that there are host-specific species or strains of *Pneumocystis*<sup>17-19</sup>. Oligonucleotides prepared to the rat *Pneumocystis* 16S-like rRNA gene recognize human *Pneumocystis*, confirming that human and rat *Pneumocystis* are closely related.

The placement of *Pneumocystis* among the Fungi raises questions regarding the acquisition of the organism and the use of chemotherapeutic agents. Fungal infections are acquired from environmental sources and the infective form of the organism is capable of growth outside the host. Protozoan parasites, on the other hand, generally require growth in a definitive host to maintain an infectious pool. *Pneumocystis* is acquired from the environment via an airborne route<sup>20,21</sup> and attempts to infect rats with isolated organisms have failed, which suggests that neither the cyst nor the trophozoite is the infective stage. This is analogous to histoplasmosis where an infective spore is acquired from environmental sources, but the yeast form which causes disease is non-infectious<sup>20</sup>. Cryptococcosis is also acquired from the environment, but the infective stage has not been determined<sup>22</sup>. None of these three infections is spread by human-to-human or animal-to-human transmission. Like these closely-related organisms, *Pneumocystis* may have a niche outside the mammalian host which produces the infective form. *P. carinii* pneumonia responds to what are generally believed to be 'anti-protozoan' drugs (pentamidine or the combination of trimethoprim and sulfamethoxazole), but, these agents also possess anti-fungal activity and are adjunctive therapy for histoplasmosis and paracoccidioidomycosis<sup>22,23</sup>. Therefore, sensitivity to these agents is not inconsistent with the fungal nature of *Pneumocystis*.

The sequence of *Pneumocystis* 16S-like rRNA and its phylogenetic analysis presented here establish the organism as a member of the Fungi. With this knowledge, the life cycle of

*Pneumocystis* can be compared to those of the Fungi rather than to Protozoa. This relationship should allow insight into the design of new chemotherapeutic agents for *Pneumocystis* as well as methods for its axenic cultivation.

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## Increased virulence of *Yersinia pseudotuberculosis* by two independent mutations

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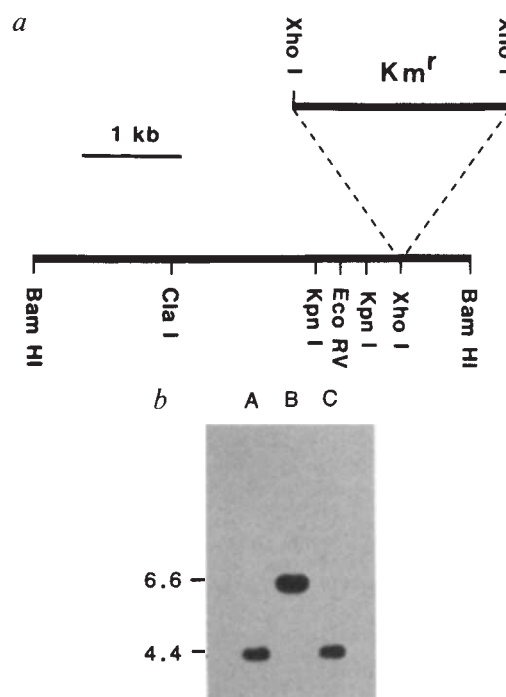
A chromosomally encoded protein, which mediates invasion into HeLa cells was recently identified in *Yersinia pseudotuberculosis*<sup>1,2</sup>. The role of this protein (invasin) in the virulence process was not, however, investigated. We show that mutation of the invasin gene in *Y. pseudotuberculosis* abolishes the ability of the bacteria to invade HeLa cells. When mice were challenged by intraperitoneal injection both the mutant and the wild-type strain produced infections of similar virulence but mutant showed a slower rate of infection after oral challenge. A double mutant, carrying an additional mutation in the gene coding for the Yop1 protein<sup>3,4</sup>, was also constructed. The double mutant was significantly more virulent than either the wild-type or the corresponding single mutants. *Y.*

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*pestis*, in contrast to *Y. pseudotuberculosis* lacks the ability to express either invasin or YopI<sup>5-7</sup>, sequence analysis of the *yopA* gene from both *Y. pestis* and *Y. pseudotuberculosis* shows that the *yopA* gene of *Y. pestis* contains a point-mutation leading to a reading-frame shift. When the *yopA*<sup>+</sup> gene was introduced into *Y. pestis* the virulence of this strain was reduced. These results may provide insight into the rise and fall of plague epidemics caused by *Y. pestis*.

The gene coding for invasin, *invA*, was cloned from the chromosomal DNA of *Y. pseudotuberculosis* strain YPIII. The isolated clone, pIRR1, contained a 4.4 kilobase (kb) *Bam*HI insert with a single *Xho*I site (Fig. 1). Insertion of a 2.2 kb *Xho*I fragment of transposon Tn5, which carries the kanamycin resistance gene, generated pIRR11 (Fig. 1). Both plasmids were introduced into the C600 strain of *Escherichia coli* and tested for their ability to invade HeLa cells (Table 1). In contrast to strain C600(pIRR1), strain C600 (pIRR11) lacked any significant invasive capability (Table 1). Analysis in a minicell system



**Fig. 1** *a*, Restriction endonuclease map of the 4.4 kb. *Bam*HI fragment carrying the structural gene of the invasin. The gene was isolated from a *Bam*HI digest of chromosomal DNA from strain YPIII cloned into the vector pACYC184, using the methods and strategy described in ref. 1. The hybrid plasmid containing the invasin gene was denoted pIRR1. Plasmid pIRR11 was constructed by insertion of the 2.2 kb *Xho*I fragment of transposon Tn5 which encodes kanamycin resistance. *b*, Southern blotting, using the purified 4.4 kb fragment encoding the invasin, as a probe, demonstrates the insertion of the 2.2 kb *Xho*I fragment into the structural gene of the invasin of strain YPIII (pIB1). Lane A, *Bam*HI digested chromosomal DNA of strain YPIII (pIB1). Lane B, *Bam*HI digested chromosomal DNA of YP100(pIB1). Lane C, *Bam*HI digest of pIRR1.

HeLa cells were seeded in a 24-well tissue culture plate at a density of  $1 \times 10^5$  cells per well in 1 ml Leibovitz L-15 medium supplemented with 10% heat inactivated fetal calf serum, and incubated at 37 °C. Bacteria were grown over night in nutrient broth on a rotary shaker at 26 °C (*Yersinia*) or 37 °C (*E. coli*) and diluted in 0.9% NaCl to a concentration of  $1 \times 10^6$  bacteria per ml. The HeLa cell cultures were infected with ten bacteria per HeLa cell and centrifuged for 5 min at 400 g. After 1 h at 37 °C the infected cultures were washed twice with 1 ml of L-15 medium and then overlaid with 1 ml of L-15 medium supplemented with 10% heat inactivated fetal calf serum and  $10 \mu\text{g ml}^{-1}$  gentamicin. After further incubation at 37 °C for 1 h the cell cultures were again washed twice. The HeLa cells were lysed by adding 1 ml 0.1% Triton X-100 in 0.9% NaCl, releasing internalized bacteria from the monolayers. The number of viable internalized bacteria was determined by plating on blood agar plates.

\* Percentage of bacteria added to HeLa cell cultures that resisted gentamicin treatment.

showed that pIRR1 but not pIRR11 directed expression of a polypeptide of relative molecular mass 103,000 ( $M_r$ 103K), the expected size of invasin<sup>2</sup> (data not shown). Thus, the *Xho*I insertion abolished expression of the 103 K polypeptide as well as the ability of the plasmid to confer the invasive phenotype upon C600.

Transformation of the virulent *Y. pseudotuberculosis* strain YPIII (pIB1) with pIRR11 led, by homologous recombination, to exchange of the *invA*<sup>+</sup> gene with the interrupted *invA* gene. Analysis of the resulting strain, YP100 (pIB1), by Southern blotting confirmed that the 2.2 kb *Xho*I fragment had been integrated into the structural gene of the invasin (Fig. 1). The mutation in the invasin gene abolished the ability, possessed by the wild-type strain YPIII(pIB1) to invade cultured HeLa cells (Table 1).

The role of invasin in pathogenesis was investigated by determining the LD<sub>50</sub> dose of YPIII(pIB1) and YP100(pIB1) after either interperitoneal (i.p.) injection or oral infection of Swiss albino mice (Table 2). For both strains the LD<sub>50</sub> after i.p. injection was approximately  $10^4$  bacteria. After oral challenge, however, strain YP100(pIB1) showed a delayed rate of infection compared to the wild-type strain. Mice infected with the wild-

type strain died after 5–6 days whereas the mutant strain killed the mice 8–10 days after infection. These results suggest that invasin may have a role in pathogenesis of *Y. pseudotuberculosis* when infection is initiated via the oral route. It is possible that invasin acts as an adhesin, and there might be another adhesion which could complement the function of the invasin. One obvious candidate would be the plasmid-encoded protein Yop1 (*yopA*<sup>+</sup>), which has recently been shown to mediate haem-agglutination of guinea-pig erythrocytes<sup>8</sup>. To test this possibility plasmid pIB103 (ref. 9), carrying transposon Tn5-132 inserted in the structural gene of Yop1, was introduced into strain YP100. Surprisingly, strain YP100 (pIB103) was more virulent than the corresponding wild-type strain and the single mutants regardless of the route of infection (Table 2). These results suggest that invasin is dispensable and may only be important as a virulence determinant when Yop1 is present. In the absence of Yop1 and the invasin, secondary structures which promote adherence to the epithelial cell layer may be unmasked. The ability of the double mutant to cause disease even after oral infection support this view.

Previous studies have indicated that virulent strains of *Y. pestis* are unable to invade HeLa cells<sup>10</sup> and it has been shown that *Y. pestis* EV76 does not express the Yop1 polypeptide<sup>5-7</sup>. Our results which demonstrate that virulence increases in the absence of both Yop1 and the invasin, could thus, in part, explain the enhanced virulence of *Y. pestis* compared to *Y. pseudotuberculosis*. Other factors are, however, likely to be involved because *Y. pestis* exhibits additional potential virulence determinants; the fraction 1 antigen, fibrinolysin, coagulase and pesticin<sup>11-15</sup>.

ATGACTAAAGATTTTAAGATCAGTGTCTCTCGCGCATTAATATCTCGTGTCTTCATCTCCATATGCATTGGCCGAGGAGCCGAGGATGGC  
 ATGACTAAAGATTTTAAGATCAGTGTCTCTCGCGCATTAATATCTCGTGTCTTCATCTCCATATGCATTGGCCGAGGAGCCGAGGATGGC  
 m t k d f k i s v s a a l i s a l f s s p y a f a e e p e d r

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 AACGATGGTATTCCTCGTTTGTTCAGCAGTTCAAATAAGCCCAATGTTGATCCTAAATGGGTGTGGGATTATATCCAGCAAAACCAATATTA  
 n d g i p r l s a v q i s p n v d p k l g v g l y p a k p i l

CGTCAAGAAAACCCAAATACCTCCACGAGTCCCAAGGTCAGAAAAAAGAGTAGATTACGAGAAGCAATACCAACCAAGTACTA  
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 r q e n p k l p p r g p q g p e k k r a r l a e a i q p q v l

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 GGCGCAGCGGGCTCAATGCTCGGCTAAGGATCCCTATAGCATTGCGATTGGTCTACTGCTGAAGCAGCAAAACCCAGCAGCAATTGCTGTG  
 g a g l n a r a k d p y s i a i g a t a e a a k p a a v a v

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 GGCTCTGGTCAATGGCAACAGCGCTTGAATCTGTGCAATGGTCTTTAAGTAAGGCATTGGGAGATTGGCAGTTACTTATGGGGTAAGT  
 g s g s i a t g v n s v a i g p l s k a l g d s a v t y g a s

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 AGTACCGCCAGAAAGATGGAGTAGCTATCGGTGCGAAGCATCAGCTTCGGTACTGCTGCTGCTGCTGCTTTAACTCGAAAGTTGATGCA  
 s t a q k d g v a i g a r a s a s d t g v a v g f n s k v d a

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 CAAACTCTGTTGCCATTGGACACTCTAGTCACGTTGGCGGAGATCATGTTTATCAATGCAATGGGGATCATTCTAAACTGACCGAGAG  
 q n s v a i g h s h s v a a d h g y s i a i g d h s k t d r e

AATAGTGTATCCATTGGTCATGAAAGCCTTAATCGCAATTAACACATCTTGGCGCTGGCAGTAAGACACTGATGCAAGTGAATGTCGCGCAA  
 AATAGTGTATCCATTGGTCATGAAAGCCTTAATCGCAATTAACACATCTTGGCGCTGGCAGTAAGACACTGATGCAAGTGAATGTCGCGCAA  
 n s v s i g h e s l n r q l t h l a a g t e d t d a v n v a q

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 TTAAAGAAAGAAATGGCTGAAACATTGGAATGCAAGTAAAGAGACTTTGGCTCAGTCTAAGCATGTTTGGATGCGGCCAAAAACACTCA  
 l k k e m a e t l e n a r k e t l a q s n d v l d a a k k h s

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 AATAGTGTGCGAGAACCACTTTAGAACTGCTGAAGAACATGCAATAAAAATCAGCTGAAACCGTTAGTAAGCGCTAAAGTGTATGCAGAC  
 n s v a r t t l e t e a e h a n k k s a e a l v s a k v y a d

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 AGCAATCTTCTCAACACTAAAACTGCAATAGCTATACCGATGTGACTGTAAGTAATTCGACTAAGAAAGCAACCGTGAATCTAATCAA  
 s n s s h t l k t a n s y t d v t v s s s t k k a i s e s n q

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 TACACAGATCATAAATTGAGTCAACTTGACAAACCGTTAGATAAACTTGACAAACGAGTTGACAAAGGTTTAGCCAGTTTACGCGCTTTAAAC  
 y t d h k f s q l d n r l d k l d k r v d k g l a s s a a l n

AGCTGTGTCAGCCATATGCTGAGGAAAGTAACTTTACTGCGAGTGTGCGGGATATCGTTTACTGAGGCATTAGCAATGGTCTGCGC  
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 s l f q p y g v g v g y g n f t a g v g g y r s s q a l a i g s g

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 TATCGTGAATGAGAGTGTGCGCACTTAAAGCCGGTGTGGCTTATGCGGGTCTCTCGAATGTCATGTACAACGCATCATTAAATATCGAGTGG  
 y r v n e s v a l k a g v a y a g s s n v m y n a s f n i e w

TAATATCATTTAGAAATTAACAAGTCTATAGGAAACACCGATTACATAATCGTAATGGTGTGTTTATTA  
 TAATATCATTTAGAAATTAACAAGTCTATAGGAAACACCGATTACATAATCGTAATGGTGTGTTTATTA  
 end

**Fig. 2** DNA sequence of the *yopA* genes of pYV019 and pIB1. Nucleotides are underlined where there are differences between the two sequences. Inverted arrows indicate a possible transcription terminator sequence. The one base deletion of pYV019 leading to a reading frame shift is marked with an arrow. The corresponding amino acid sequence is given in one letter code and the translation stop codon is indicated by 'end'. The underlined amino acid sequence is identical to the N-terminal amino acid sequence obtained from the purified Yop1 polypeptide<sup>4</sup>.

**Methods.** DNA sequencing was performed using the M13 dideoxy chain termination method<sup>25</sup>. Appropriate M13 templates were obtained by cloning DNA fragments derived from the cloned genes<sup>16</sup>.

Our own results confirm that *Y. pestis* strain EV76 does not penetrate HeLa cells, and show that introduction of pIRR1 confers the invasion phenotype, demonstrating that invasins can be functionally expressed in *Y. pestis* (data not shown). Southern blotting analysis using pIRR1 as a probe revealed no positive signal, supporting the conclusion that *Y. pestis* is unable to express invasins.

Recently we demonstrated that the EV76 plasmid pYV019 contains genetic information homologous to the *yopA*<sup>+</sup> gene of pIB1 (ref. 16). To increase our understanding of the evolution of the two *Yersinia* species and also to gain insight into the role of the Yop1 protein in pathogenesis, we sequenced the two *yopA* genes (Fig. 2). The results revealed one significant difference, a point mutation in strain pYV019 which results in a reading frame shift and truncation of the *yopA* gene product (Fig. 2). This would explain why *Y. pestis* is unable to express the Yop1 protein. The *yopA*<sup>+</sup> gene of pIB1 was found to encode a protein having a molecular weight of 42.7 K with sequence matching the NH<sub>2</sub>-terminal amino acid sequence obtained from purified Yop1 protein<sup>4</sup>. Downstream of the structural gene a possible transcription terminator was identified. Thus, the point mutation in pYV019 is unlikely to have a polarity effect on downstream genes. The deletion has occurred in a stretch of DNA that contains nine deoxyadenosine nucleotides. Such regions of redundancy are known to be hot-spots for mutation<sup>17</sup> and it is therefore possible that this region of the *yopA* gene is subjected to an increased mutation rate.

**Table 2** LD<sub>50</sub> doses of different strains of *Y. pseudotuberculosis* after infection of Swiss albino mice

| Strain         | Relevant marker                      | LD <sub>50</sub>      |                       |
|----------------|--------------------------------------|-----------------------|-----------------------|
|                |                                      | i.p.                  | Oral                  |
| YPIII (pIB1)   | wild-type                            | 1 × 10 <sup>4</sup>   | 5 × 10 <sup>9</sup>   |
| YP100 (pIB1)   | Inv <sup>-</sup> , Yop1 <sup>+</sup> | 1 × 10 <sup>4</sup>   | 2 × 10 <sup>9</sup>   |
| YPIII (pIB103) | Inv <sup>+</sup> , Yop1 <sup>-</sup> | 2 × 10 <sup>4</sup>   | 1 × 10 <sup>9</sup>   |
| YP100 (pIB103) | Inv <sup>-</sup> , Yop1 <sup>-</sup> | 2 × 10 <sup>4</sup> * | 5 × 10 <sup>7</sup> * |
| YPIII          | plasmid <sup>-</sup>                 | 4 × 10 <sup>6</sup>   | ≥ 10 <sup>10</sup>    |

The bacteria were incubated over night at 26 °C and diluted in 0.9% NaCl to give appropriate concentrations for the LD<sub>50</sub> determinations via interperitoneal injections (i.p.). Oral infections were carried out as described by Gemski *et al.*<sup>21</sup>. In brief the bacteria were grown over night at 26 °C and diluted to the appropriate infectious dose in sterile drinking water. Swiss albino mice were kept without drinking water before being allowed to drink the infected water *ad libitum* for 16 h after which time fresh water was given. The LD<sub>50</sub> was determined according to Reed and Muench<sup>22</sup> using four groups of three mice, challenged with successive 10-fold dilutions of the different strains. The LD<sub>50</sub> determinations were repeated two or three times. A logit regression model<sup>23</sup> with common slope and different intercepts fitted well with the LD<sub>50</sub> data. The groups were further compared by *t*-tests (double-sided, 5% level) for differences between intercepts.

\* LD<sub>50</sub> values of strain YP100 (pIB103) were significantly different from the other strains tested.



**Table 3** Decreased virulence of a *yopA*<sup>+</sup> strain of *Y. pestis*

| Strain           | Relevant genotype        | LD <sub>50</sub> (i.p.) |
|------------------|--------------------------|-------------------------|
| <i>Y. pestis</i> |                          |                         |
| EV76-c (pIB1)    | <i>yopA</i> <sup>+</sup> | 6 × 10 <sup>2</sup> *   |
| EV76-c(pIB102)   | <i>yopA</i> ::Tn5        | ≤10                     |
| EV76             | wild-type                | ≤10                     |
| EV76-c           | plasmid <sup>-</sup>     | 5 × 10 <sup>6</sup>     |

The wild-type plasmid pIB1 of *Y. pseudotuberculosis* and its derivative pIB102 was introduced into the cured *Y. pestis* strain EV76-c by transduction using phage P1. The virulence of the strains listed was determined by i.p. injection. One hour before challenge the mice received 0.4 mg of Fe<sub>2</sub>SO<sub>4</sub>(7H<sub>2</sub>O) emulsified in peanut-oil, by intraperitoneal injection<sup>24</sup>. The statistical methods used are described in legend to Table 2.

\* The LD<sub>50</sub> value of strain EV76-c(pIB1) is significantly different from the other strains.

Our results suggested that introduction of the *yopA*<sup>+</sup> gene into *Y. pestis* would decrease the virulence of this strain. We therefore introduced the plasmids pIB1 (*yopA*<sup>+</sup>) and pIB102 (*yopA*::Tn5, ref. 4) of *Y. pseudotuberculosis* into the cured strain EV76-c and determined their effects on virulence (Table 3). We found that strain EV76-c (pIB1) showed an increased LD<sub>50</sub> value compared to the isogenic strain EV76-c(pIB102). As these two strains differ only in their ability to express the Yop1 protein, we conclude that expression of the Yop1 protein in *Y. pestis* reduces the virulence of the pathogen. It is possible that the results presented here can provide an explanation for observations indicating the existence of strains of *Y. pestis* of lower virulence<sup>18-20</sup> and also for the sudden appearance of plague epidemics. The endemic host of *Y. pestis* may have harboured a strain of lower virulence, which by one mutation could have become hypervirulent. The reverse mechanism would also explain the decline and termination of outbreaks. This hypothesis is at present under investigation.

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## Homology of perforin to the ninth component of complement (C9)

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Perforin is one of the cytolytic factors present in the cytoplasmic granules of mouse cytotoxic T lymphocytes and natural killer cells<sup>1-3</sup>. We have determined the sequence of the N-terminal amino acids of perforin purified from a mouse natural killer cell line, and, by using oligonucleotide probes corresponding to the amino acid residues, we have identified a complementary DNA encoding perforin from the cDNA library of a mouse cytotoxic T lymphocyte clone. As predicted from the functional similarities between perforin and the ninth component of the serum cytolytic system, complement (C9) (refs 4-8), the deduced primary structure of perforin has homology with C9 at their respective functionally conserved regions<sup>9</sup>. We find that perforin is only expressed in killer cell lines, and not in helper T lymphocytes or other tumour cells tested. Thus we have provided direct molecular evidence that a killer-cell-specific protein evolutionally linked to C9 is involved in cell-mediated cytotoxicity.

Perforin, which is contained in the cytoplasmic granules of mouse killer cells<sup>10</sup>, is known to be important in the granular exocytosis model for target cell destruction by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells<sup>11</sup>. Perforin binds to target membranes in the presence of Ca<sup>2+</sup>, and polymerizes to form transmembrane channels<sup>1-3</sup>. These channels are responsible for the osmotic lysis of the target cells. It has been proposed that perforin and complement C9 have striking similarities in terms of their structure and function<sup>4-8</sup>. But it is unclear whether these two proteins originated from a common ancestor, because of lack of information about the molecular characteristics of perforin<sup>12</sup>.

We purified perforin from a large granular lymphocyte (LGL; NK-like) cell line of mice, SPB2.4, as described previously<sup>13</sup>, and determined 31 amino acid residues of the N-terminal

N-TERMINUS: PCYTATRSECKQKHKFVPGVWVMAEGMDVTT

Probe : TGGATGGCIGGIGAGGATGGA<sup>G</sup>GT

**Fig. 1** N-terminal sequence of perforin. Perforin was purified from the mouse LGL (NK-like) cell line SPB2.4 as described<sup>13</sup>. Purified perforin was reduced and alkylated, and the N-terminal sequence was determined by automated Edman degradation using Applied Biosystems 477A protein sequencer equipped with a model 120A on-line PTH analyser. Four mixtures of oligonucleotide probes with two degenerate bases were synthesized to correspond to amino acid residues 21-29 (underlined).

sequence by microsequencing (Fig. 1). We screened the cDNA library prepared from mouse CTL clone 2C (ref. 14), which is reported to contain haemolytically active granules<sup>15</sup>, by using a mixture of four 26-mer oligonucleotides corresponding to amino acid residues 21-29 of the probe (Fig. 1). Of 2 × 10<sup>5</sup> plaques screened, five positive clones were found. The amino acid sequence deduced from the nucleotide sequence of these cDNA inserts is shown in Fig. 2. The mature perforin protein therefore probably consists of 534 amino acids, with a leader peptide of 20 amino acids. The calculated relative molecular

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|                                                                                                                                 |     |
|---------------------------------------------------------------------------------------------------------------------------------|-----|
| ATG GCC ATG TGC CTG TTC CTC CTG GGC CTT TTC CTG CTG CTG CCA CGA CCT GTC CCT GCT CCC TGC TAC ACT GCC ACT CGG TCA GAA TGC         |     |
| Met Ala Met Cys Leu Phe Leu Leu Gly Leu Phe Leu Leu Leu Pro Arg Pro Val Pro Ala Pro Cys Tyr Thr Ala Thr Arg Ser Glu Cys         |     |
| -20                                                                                                                             | -10 |
| AAG CAG AAG CAC AAG TTC GTG CCA GGT GTA TGG ATG GCT GGG GAA GGC ATG GAT GTG ACT ACC CTC CGC CGC TCC GGC TCC TTC CCA GTG 180     |     |
| Lys Gln Lys His Lys Phe Val Pro Gly Val Trp Met Ala Gly Glu Gly Met Asp Val Thr Thr Leu Arg Arg Ser Gly Ser Phe Pro Val 40      |     |
| AAC ACA CAG AGG TTC CTG AGG CCT GAC CGC ACC TGC ACC CTC TGT AAA AAC TCC CTA ATG AGA GAC GCC ACA CAG CGC CTA CCT GTG GCA         |     |
| Asn Thr Gln Arg Phe Leu Arg Pro Asp Arg Thr Cys Thr Leu Cys Lys Asn Ser Leu Met Arg Asp Ala Thr Gln Arg Leu Pro Val Ala 70      |     |
| ATC ACC CAC TGG CGG CCT CAC AGC TCA CAC TGC CAG CGT AAT GTG GCC GCA GCC AAG GTC CAC TCC ACG GAG GGT GTG GCC CGG GAG GCA 360     |     |
| Ile Thr His Trp Arg Pro His Ser His Cys Gln Arg Asn Val Ala Ala Lys Val His Ser Thr Gly Glu Val Ala Arg Glu Ala 100             |     |
| GCT GCT AAT ATC AAT AAC GAC TGG CGT GTG GGG CTG GAT GTG AAC CCT AGG CCA GAG GCA AAC ATG CGC GCC TCC GTG GCT GGC TCC CAC         |     |
| Ala Ala Asn Ile Asn Asn Asp Trp Arg Val Gly Ser Leu Asp Val Asn Pro Arg Pro Glu Ala Asn Met Arg Ala Ser Val Ala Gly Ser His 130 |     |
| TCC AAG GTA GCC AAT TTT GCA GCT GAG AAG ACC TAT CAG GAC CAG TAC AAC TTT AAT AGC GAC ACA GTA GAG TGT CGC ATG TAC AGT TTT 540     |     |
| Ser Lys Val Ala Asn Phe Ala Ala Glu Lys Thr Tyr Gln Asp Gln Tyr Asn Phe Asn Ser Asp Thr Val Glu Cys Arg Met Tyr Ser Phe 160     |     |
| CGC CTG GTA CAA AAA CCT CCA CTC CAC CTT GAC TTC AAA AAG GCG CTC AGA GCC CTC CCC CGC AAC TTT AAC AGC TCC ACA GAG CAT GCT         |     |
| Arg Leu Val Gln Lys Pro Pro Leu His Leu Asp Phe Lys Lys Ala Leu Arg Ala Leu Pro Arg Asn Phe Asn Ser Ser Thr Glu His Ala 190     |     |
| TAC CAC AGG CTC ATC TCC TCC TAT GGC ACG CAC TTT ATC ACG GCT GTG GAC CTC GCT GGC CGC ATC TCG GTC CTT ACA GCC CTG CGT ACC 720     |     |
| Tyr His Arg Leu Ile Ser Ser Tyr Gly Thr His Phe Ile Thr Ala Val Asp Leu Gly Gly Arg Ile Ser Val Leu Thr Ala Leu Arg Thr 220     |     |
| TGT CAG CTG ACC CTG AAT GGG CTC ACA GCT GAT GAG GTA GGA GAC TGC CTG AAC GTG GAG GCC CAG GTC AGC ATC GGT GCC CAA GCC AGC         |     |
| Cys Gln Leu Thr Leu Asn Gln Leu Thr Ala Asp Glu Val Gly Asp Cys Leu Asn Val Glu Ala Gln Val Ser Ile Gly Ala Gln Ala Ser 250     |     |
| GTC TCC AGT GAA TAC AAA GCT TGT GAG GAG AAG AAG AAA CAG CAC AAA ATG GCC ACC TCT TTC CAC CAG ACC TAC CGT GAG CGT CAC GTC 900     |     |
| Val Ser Ser Glu Tyr Lys Ala Cys Glu Glu Lys Lys Lys Gln His Lys Met Ala Thr Ser Phe His Gln Thr Tyr Arg Glu Arg His Val 280     |     |
| GAA GTA CTT GGT GGC CCT CTG GAC TCC ACG CAT GAT CTG CTC TTC GGG AAC CAA GCT ACA CCA GAG CAG TTC TCA ACC TGG ACA GCC TCA         |     |
| Glu Val Leu Gly Gly Pro Leu Asp Ser Thr His Asp Leu Leu Phe Gly Asn Gln Ala Thr Pro Glu Gln Phe Ser Thr Trp Thr Ala Ser 310     |     |
| CTG CCC AGC AAC CCT GGT CTG GTG GAC TAC AGC CTG GAG CCC CTG CAC ACA TTA CTG GAA GAA CAG AAC CCG AAG CGG GAG GCT CTG AGA 1080    |     |
| Leu Pro Ser Asn Pro Gly Leu Val Asp Tyr Ser Leu Glu Pro Leu His Thr Leu Leu Glu Glu Gln Asn Pro Lys Arg Glu Ala Leu Arg 340     |     |
| CAG GCT ATC AGC CAT TAT ATA ATG AGC AGA GCC CGG TGG CAG AAC TGT AGC AGG CCC TGC AGG TCA GGC CAG CAT AAG AGT AGC CAT GAT         |     |
| Gln Ala Ile Ser His Tyr Ile Met Ser Arg Ala Arg Trp Gln Asn Cys Ser Arg Pro Cys Arg Ser Gly Gln His Lys Ser His Asn Asp 370     |     |
| TCA TGC CAG TGT GAG TGC CAG GAT TCA AAG GTC ACC AAC CAG GAC TGC TGC CCA CGA CAG AGG GGC TTG GCC CAT TTG GTG GTA AGC AAT 1260    |     |
| Ser Cys Gln Cys Glu Cys Gln Asp Ser Lys Val Thr Asn Gln Asp Cys Cys Pro Arg Gln Arg Gly Leu Ala His Leu Val Val Ser Asn 400     |     |
| TTC CGG GCA GAA CAT CTG TGG GGA GAC TAC ACC ACA GCT ACT GAT GCC TAC CTA AAG GTC TTC TTT GGT GGC CAG GAG TTC AGG ACC GGT         |     |
| Phe Arg Ala Glu His Leu Trp Gly Asp Tyr Thr Thr Ala Thr Asp Ala Tyr Leu Lys Val Phe Phe Gly Gly Gln Glu Phe Arg Thr Gly 430     |     |
| GTC GTG TGG AAC AAT AAC AAT CCC CGG TGG ACT GAC AAG ATG GAC TTT GAG AAT GTG CTC CTG TCC ACA GGG GGA CCC CTC AGG GTG CAG 1440    |     |
| Val Val Trp Asn Asn Asn Asn Pro Arg Trp Thr Asp Lys Met Asp Phe Glu Asn Val Leu Leu Ser Thr Gly Gly Pro Leu Arg Val Gln 460     |     |
| GTC TGG GAT GCC GAC TAC GGC TGG GAT GAT GAC CTT CTT GGT TCT TGT GAC AGG TCT CCC CAC TCT GGT TTC CAT GAG GTG ACA TGT GAG         |     |
| Val Trp Asp Ala Asp Tyr Gly Trp Asp Asp Asp Leu Leu Gly Ser Cys Asp Arg Ser Pro His Ser Gly Phe His Glu Val Thr Cys Glu 490     |     |
| CTA AAC CAC GGC AGG GTG AAA TTC TCC TAC CAT GCC AAG TGT CTG CCC CAT CTC ACT GGA GGG ACC TGC CTG GAG TAT GCC CCC CAG GGG 1620    |     |
| Leu Asn His Gly Arg Val Lys Phe Ser Tyr His Ala Lys Cys Leu Pro His Leu Thr Gly Gly Thr Cys Leu Glu Tyr Ala Pro Gln Gly 520     |     |
| CTT CTG GGA GAT CCT CCA GGA AAC CGC AGT GGG GCT GTG TGG TAA                                                                     |     |
| Leu Leu Gly Asp Pro Pro Gly Asn Arg Ser Gly Ala Val Trp End                                                                     |     |

**Fig. 2** Nucleotide sequence and deduced amino acid sequence of perforin cDNA. Nucleotides are numbered in the 5' to 3' direction, with amino acids below the sequences. Numbering of amino acids starts at the first residue of the N-terminal sequence of perforin. Nucleotides hybridized with oligonucleotide probes are boxed. Solid lines, Asn-linked potential *N*-glycosylation sites.

**Methods.** A cDNA library constructed from the mouse CTL clone 2C in the phage vector  $\lambda$  gt10 (ref. 14) was screened with a mixture of ( $^{32}$ P) end-labelled oligonucleotide probes corresponding to the N-terminal amino acids 21–29 (Fig. 1). Hybridization was carried out at 65 °C for 16 h in 6×SSPE, 2×Denhardt's solution and 0.05% sodium pyrophosphate containing 200  $\mu$ g ml $^{-1}$  denatured salmon sperm DNA. Filters were washed three times for 10 min at 65 °C in 6×SSPE and 0.05% sodium pyrophosphate, dried and exposed to Kodak XAR-5 film with an intensifying screen at –80 °C for 24 h. Positive plaques were re-screened and tertiary-screened with the mixed probe. Perforin cDNA inserts were subcloned into the *Eco*RI site of Bluescript plasmid (Stratagene), and deletion mutant clones were prepared by unidirectional digestion with exonuclease III and mung bean nuclease as described previously<sup>27</sup>. All nucleotide sequences were determined on both strands of the cDNA by the dideoxynucleotide termination method<sup>28</sup> using Sequenase (USB).

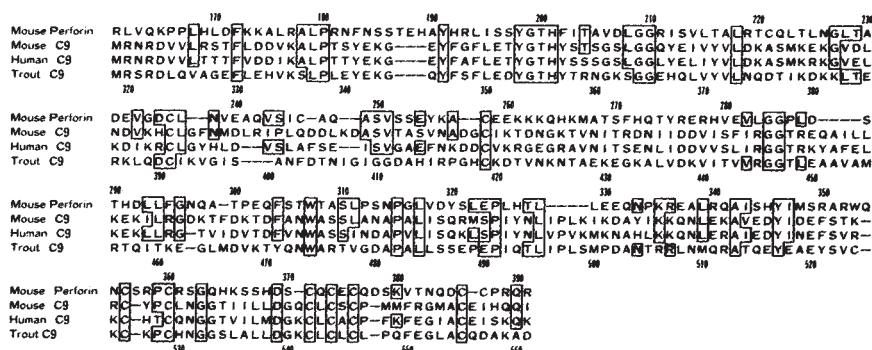
mass ( $M_r$ ) of the predicted mature protein is 59,919 and it contains three potential *N*-glycosylation site. Post-translational modification with carbohydrate at these sites may account for the  $M_r$  of the perforin molecule present in the cytoplasmic granule, which has been shown to be 70,000 (70K) as described previously<sup>13</sup>. The known N-terminal sequence of purified perforin is identical to the prediction from the cDNA sequence. Hydrophobicity plot analysis reveals no stretches of obviously hydrophobic regions.

Comparison of the amino acid sequences of perforin and the C9s of mouse<sup>9</sup>, human<sup>16,17</sup> and trout<sup>9</sup> reveals no significant overall homology between perforin and these C9 sequences, but perforin residues 170–390 do resemble C9 residues 328–560 (numbering given is for mouse) (Fig. 3). This region of C9 is believed to contain two functionally important domains. The first domain, the putative membrane-spanning region of C9 (ref. 9) corresponds to residues 176–210 of perforin. As both proteins should be able to construct the transmembrane channel, the

conservation of this region between perforin and C9 is expected. Indeed, perforin residues 190–203 can be folded into the amphipathic  $\alpha$ -helix structure as previously shown for C9 (ref. 9). The other homologous region is the second cysteine-rich domain of C9, also known as the EGF-type 'class B' cysteine-rich region<sup>18</sup>, which corresponds to perforin residues 356–386. The conserved six cysteines present in this region may form intramolecular disulphide bonds and contribute towards maintaining a functionally important structure. These two homologous regions are also conserved in C8 $^a$  (ref. 19) and C8 $^b$  (refs 20, 21). These findings support the proposal that perforin is a member of the protein family that includes C9 and C8. Perforin lacks the 'class A' LDL receptor-homologous region corresponding to the first cysteine-rich domain in the N-terminus of C9 (residues 77–114 in mouse C9); however, it does have a unique C-terminal portion of residues 410–534. These structural differences between perforin and C9 reflect the functional differences between them. For example, C9 interacts with C5b–8 to insert into the mem-

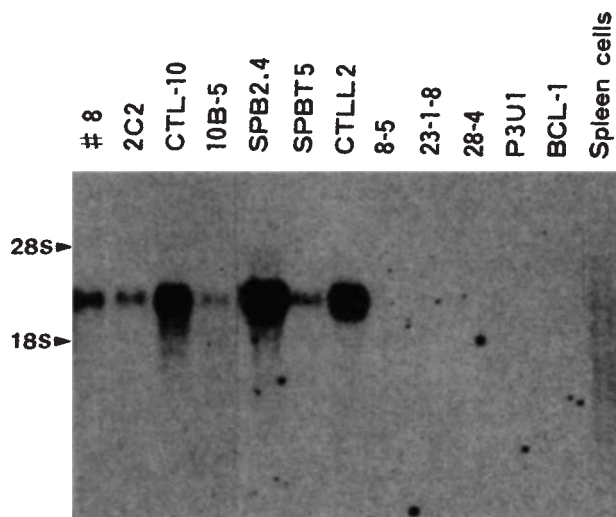


**Fig. 3** Alignment of amino acid sequences of perforin with complement mouse<sup>9</sup>, human<sup>16,17</sup> and trout<sup>9</sup> C9. Amino acid residues identical to perforin residues are boxed. The numbering of the upper panel corresponds to the perforin sequence and the lower to the mouse C9 sequence.



**Fig. 4** Northern blot analysis of perforin mRNA. The following mouse cell lines were analysed; LGL cell lines<sup>29</sup>: SPB2.4(BALB/c), SPBT5(BALB/c); CTL cell lines<sup>30</sup>: CTLL2 (C57BL/6), 2C2 (C57BL/6 anti FBL-3), #8 (C57BL/6 anti FBL-3), CTL-10 (CB6F1 anti UV $\delta$ 1 sarcoma), 10B-5 (CB6F1 anti UV $\delta$ 1 sarcoma); helper T cell lines<sup>31</sup>: 23-1-8, 28-4, 8-5 (all B6C3F1 anti-KLH); YAC-1 (thymic lymphoma, A), P3U1 (myeloma, BALB/c), BCL-1 (B cell leukaemia lymphoma, BALB/c).

**Methods.** Cytoplasmic RNA from  $1 \times 10^6$  cells were prepared<sup>32</sup>, electrophoresed on a 1% formaldehyde-agarose gel, transferred to a Nytron membrane (S&S), and hybridized with <sup>32</sup>P-UTP-labelled riboprobe containing the coding sequence of perforin by the Blue-script T7 RNA transcription system (Stratagene). Hybridization was performed at 65°C for 12 h in 50% formamide, 6 $\times$ SSPE, 0.5% SDS, 2.5 $\times$ Denhardt's solution containing 200  $\mu$ g ml<sup>-1</sup> of denatured salmon sperm DNA. Filters were washed four times for 15 min at 65°C in 0.1 $\times$ SSPE containing 0.1% SDS and exposed at -80°C for 8 h.



brane, but perforin by itself can polymerize and form membrane channels in the presence of Ca<sup>2+</sup> (ref. 6).

Unlike C9, perforin is thought to be a Ca<sup>2+</sup>-binding protein, as Ca<sup>2+</sup> is essential for forming the membrane channel<sup>2,10</sup>. But we could not find the typical E-F hand structure<sup>22</sup> that is conserved in Ca<sup>2+</sup>-binding proteins such as calmodulin<sup>23</sup> or troponin-c<sup>24</sup>, in the predicted primary structure of perforin.

We examined whether perforin is produced exclusively in the cells exhibiting killer activity using Northern blot analysis on several cell lines, probing with the cloned perforin cDNA (Fig. 4). A transcript of ~3.0 kb was detected in all the CTL lines (CTLL2, 2C2, #8, CTL-10 and 10B-5) and NK-like cell lines (SPB2.4 and SPBT5) examined. The amounts of transcript revealed are closely correlated with the haemolytic activities of the cytoplasmic granules prepared from these killer cell lines; killer cells SPBT5, #8, 2C2 and 10B-5 were haemolytically negative in a small preparation assay (data not shown). All of the killer cell lines tested did express the perforin gene, albeit to different extents, but no transcript was detected for several helper T cell lines or other tumour cell lines. This result indicates that perforin is expressed in a killer-cell-specific manner and supports the concept that all killer cells may be able to use perforin to lyse target cells. The expression of perforin messenger RNA may be restricted to activated killer cells, as little, if any, of the transcript was detected in normal mouse spleen cells (Fig. 4).

It is still controversial whether human perforin is also involved in cell-mediated cytotoxicity<sup>25,26</sup>. We have recently identified the human homologue of mouse perforin in human killer cells using cross-hybridization with the mouse cDNA (manuscript in preparation).

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## The molecular evolution of genes and proteins: a tale of two serines

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The advent of techniques for cloning and rapidly sequencing DNA has produced an explosive increase of sequence information for nucleic acids and their inferred proteins. Careful study of this large store of data might give us new insights into the relations between the linear sequences of genes and their functions embodied in the three-dimensional structure of proteins, and also illuminate the origin and evolution of the structural complexity of present-day proteins. Here I argue from such a study that the active site sequences of enzymes that have analogous essential serine residues lie in fact on two lines of descent from an ancient ancestral enzyme which had a cysteine instead of serine in its active site. This is based on the assumption that the two codon types which define the separate lines of descent and which have different bases in two positions could not interconvert by single mutations.

It is unlikely that there is some automatic procedure which could penetrate and unmask the complexity produced by billions of years of natural selection. Some theory is needed if only as a guide and I therefore sketch very briefly the general ideas that form the background to this work. First, I assume that the earliest proteins were small peptides of about ten amino acids, and specified by small primitive genes, probably made of RNA. In general, peptides of this length do not have a precise structure and the chemical functionality of amino acid side chains is very limited. However, peptides that included amino acids such as cysteine, histidine, aspartate and glutamate could associate with metal ions like iron, manganese, zinc, copper, magnesium and calcium and some of these sequences might have had the right stereochemistry to form stable and catalytically active complexes. Their genes would have undergone natural selection, especially if these primitive enzymes catalysed a step in gene replication or expression. In the next stage, I postulate that the genes become joined together at random and a primitive splicing mechanism concatenates the peptides into longer molecules. The principle of local functionality—the dependence of the activity on a short linear stretch of sequence—ensures that the discrete functions of the individual peptides would continue undisturbed in the composite molecule. The model sees the elemental peptides as the forerunners of present day exons and implies that genes were highly interrupted from the very beginning. Exon exchanges between the composites accompanied by mutations might then produce new molecules with useful interactions between the different peptide components. Improved and new functions could therefore evolve in a continuous and incremental manner. Eventually the association of function with local sequence would, on the whole, be lost as new active sites become formed by interactions between distant regions of the polypeptide chain. This process produced compact protein domains with a characteristic size of about a hundred amino acids from which, by further concatenation, more complex proteins were then assembled.

Relics of the original peptides may exist in modern sequences, particularly in the active sites of enzymes. However, in most metallo-enzymes, the amino acids that bind metals are located in different parts of the polypeptide chain, so that the metal ion sits in a cage formed by the three-dimensional structure, or, as in the haem proteins, in a porphyrin, which itself may have evolved to replace an original iron-binding peptide. However, local bonding has been preserved in the zinc fingers<sup>1</sup> and calcium-binding domains<sup>2</sup>, which also correspond to exons in present-day proteins<sup>3,4</sup>. These might well be good examples of peptides that have evolved without much change, even though

their present function is regulatory rather than catalytic. But there are many enzymes that do not have metals in their catalytic centres, and there are also some in which the functional amino acids have no metal-binding properties. We must then assume that these active sites evolved from earlier ones. Is it possible to find evidence for this in present-day proteins? In this paper I show that there is such evidence and that it allows a plausible reconstruction of part of the pathway of molecular evolution.

The analysis depends on a special feature of the genetic code. Several amino acids have multiple codon representations, but serine is unique in that its two sets of codons, TCN and AGY, cannot be converted into each other by single nucleotide mutations. The representations are connected by two other codons, ACN, for threonine, and TGY, for cysteine. We can therefore look at serines in highly conserved sequences of present-day proteins, and ask whether these have the same or different codon representations, and, by implication, common or different paths of descent. The critical examples are the hydrolytic enzymes with serine as an essential part of the active site, because the requirement for a specific function exerts a special constraint, which allows us to say something about the path of descent.

We have many sequences for several classes of active serine enzymes. Table 1 lists the active site sequences and their serine codons. The remarkable result is that, with the exception of the serine proteases of the subtilisin class, both representations of serine, TCN and AGY, are found in what are otherwise highly conserved sequences. In the penicillin-reactive enzymes and proteins, even the beta-lactamases, which have very similar overall sequences, have both kinds of serine; interestingly, TCN is found in the enzymes from Gram-positive bacteria, whereas AGY is used in those from Gram-negatives. The active site sequences of alkaline phosphatase also have two kinds of serine. Both serines are found amongst esterases, lipases and related enzymes; in particular, the acetylcholinesterase of *Drosophila* has TCG while in the closely related enzyme from *Torpedo*, the active serine is AGT. An even more informative example is provided by the serine proteases. Their active site sequences allow us to distinguish two classes: one, like trypsin, has TCN while the other, like thrombin, has AGY. What is most striking is the absolute correlation of the type of serine with the local intron structure of the gene. We have 17 sequences of independent genes of the TCN class and four of the AGY class; the probability of getting this pattern if there was a random association is about 1 in 2 million ( $2^{-21}$ ).

This proves that there have been at least two different lines of descent for the active site sequences of the serine proteases and it is reasonable to extend this to all of the enzymes. The simplest pathway for this convergent evolution is by the divergence of each line from a precursor that was itself catalytically active and had much the same sequence. The codons that connect the two serines are ACN for threonine, or TGY for cysteine, and, of these, cysteine is much more probable as the predecessor with catalytic function. In fact, there is some evidence for this in the case of a beta-lactamase, where replacement of the serine by cysteine gives an enzyme with some, albeit low, activity<sup>5</sup>, whereas the threonine substituted enzyme is totally inactive<sup>6</sup>. A thiol-subtilisin, made by chemical substitution, lost all of its activity for specific substrates, but retained considerable reactivity for nitrophenyl esters<sup>7</sup>. We therefore conclude that the precursor of all these serine active sites was the corresponding cysteine active sequence; so that, for example, all of the serine proteases were once cysteine proteases. The precise correlation between exon structure and serine representation in the mammalian serine proteases shows that the two lines of descent were associated with two different pieces of DNA which were either different to begin with or altered their structure shortly after the divergence. Even more remarkable is the possibility that the acetylcholinesterase of insects could have originated separately from that of vertebrates, but we need more sequence information to be certain of this.



Table 1 Sequences and serine codons of active sites

| (I) Penicillin-reactive proteins |              |     | (IV) Esterases and lipases (cont.) |                             |     |
|----------------------------------|--------------|-----|------------------------------------|-----------------------------|-----|
| D-alanine carboxypeptidase       | *            | Ser | Transacylase                       |                             |     |
| PB5 <i>E. coli</i>               | DPASLT KMMT  | AGC | LC, human                          | VFLIGHSLGCL                 | AGC |
| PB6 <i>E. coli</i>               | DPASLT KIMT  | AGC | FAS, yeast                         |                             |     |
| <i>Streptomyces</i> R61          | RVGSVTKSFS   | AGC | Acetyl transacylase                | KGATGHSQGLV                 | TCT |
| Beta-lactamase                   |              |     | Malonyl transacylase               | ATFAGHSLGEY                 | TCT |
| TEM tn3                          | PMMSTFKVLL   | AGC | Related proteins                   |                             |     |
| <i>K. pneumoniae</i>             | PMVSTFKVLL   | AGC | Vitellogenin                       | *                           |     |
| <i>R. capsulata</i>              | LMNSTVKVPV   | AGC | YP1 <i>Drosophila</i>              | IHLIGQNVGAH                 | AAT |
| <i>P. aeruginosa</i>             | PLNSTVKAFS   | AGC | YP2 <i>Drosophila</i>              | IHLIGSGPAAH                 | GGA |
| <i>Streptomyces albus</i>        | PMCSVFKTLS   | TCG | YP3 <i>Drosophila</i>              | IHLIGQGISAH                 | GGA |
| <i>B. licheniformis</i>          | AFASTIKALT   | TCG | Thyroglobulin                      | VTLAADRGGAD                 | CGT |
| I <i>B. cereus</i>               | AFASTYKALA   | TCA |                                    |                             |     |
| III <i>B. cereus</i>             | AFASTSKSLA   | TCT |                                    |                             |     |
| <i>Staph. aureus</i>             | AYASTSKAIN   | TCA |                                    |                             |     |
| Oxacillinase                     |              |     | (V) Serine proteases               | In                          | Ser |
| OXA-2                            | SPASTFKIPH   | TCG | Trypsin, rat                       | SCQ <sup>a</sup> GDSGGP VVC | TCT |
| OXA-1                            | APDSTFKIAL   | TCA | Chymotrypsin, rat                  | SCM <sup>a</sup> GDSGGP LVC | TCC |
| PSE-2                            | LPASTFKIPN   | TCA | Elastase I, rat                    | GCQ <sup>a</sup> GDSGGP LHC | TCT |
| <i>ampC</i>                      |              |     | Elastase II, rat                   | SCN <sup>a</sup> GDSGGP LNC | TCC |
| <i>E. coli</i>                   | ELGSVSKTFT   | TCG | Elastase III, human                | GCN <sup>a</sup> GDSGGP LNC | TCT |
| <i>C. freundii</i>               | ELGSVSKTFT   | TCG | CF B, human                        | TCT <sup>a</sup> GDSGGP LIV | TCT |
| <i>blaR1</i> regulatory protein  |              |     | Factor XII, human                  | ACQ <sup>a</sup> GDSGGP LVC | TCC |
| <i>B. licheniformis</i>          | APASTYKVFS   | TCC | TPA, human                         | ACQ <sup>a</sup> GDSGGP LVC | TCC |
| Transglycolase-transpeptidase    |              |     | UPA, human                         | SCQ <sup>a</sup> GDSGGP LVC | TCA |
| PBP1A <i>E. coli</i>             | QVGSNIKPFL   | TCC | Factor XI, human                   | ACK <sup>a</sup> GDSGGP LSC | TCG |
| PBP1B <i>E. coli</i>             | SIGSLAKPAT   | TCG | NGF alpha, mouse                   | TCK <sup>a</sup> GDSGGP LIC | TCA |
| PBP3 <i>E. coli</i>              | EPGSLVKPMV   | TCA | NGF gamma, mouse                   | TCE <sup>a</sup> HDSGGP LIC | TCA |
| (II) Alkaline phosphatase        | *            |     | Kallikrein, mouse, mGK-1           | TCV <sup>a</sup> GDSGGP LIC | TCA |
| Human placenta                   | VPDSGATATA   | AGT | Kallikrein, mouse, mGK-2           | TCK <sup>a</sup> GDSGGP LIC | TCA |
| Human intestine                  | VPDSAATATA   | AGC | Kallikrein, mouse, mGK-5           | TCA <sup>a</sup> GDSGGP LIC | TCA |
| <i>PHO8</i> yeast                | VTDSAAGATA   | TCA | Adipsin, mouse                     | TCT <sup>a</sup> GDSGGP LVC | TCC |
| <i>E. coli</i>                   | VTDSAASATA   | TCG | MCP II, rat                        | AFM <sup>a</sup> GDSGGP LLC | TCT |
| (III) Subtilisin proteases       | *            |     | CCPI, mouse                        | SFR <sup>a</sup> GDSGGP LIS | TCT |
| <i>B. licheniformis</i>          | NGTSMASPHV   | TCA | CCPII, mouse                       | SFE <sup>a</sup> GDSGGP LIS | TCT |
| <i>B. amyloliquefaciens</i>      | NGTSMASPHV   | TCA | CCP, mouse                         | SCN <sup>a</sup> GDSGGP LLC | TCT |
| <i>Serratia</i>                  | SGTSMASPHV   | TCA | PP, human                          | GCN <sup>a</sup> GDSGGP LNC | TCT |
| <i>Strep. cremoris</i>           | SGTSMASPHV   | TCA | PAE, dog                           | TCK <sup>a</sup> GDSGGP LIC | TCA |
| <i>Thermus aquaticus</i>         | NGTSMATPHV   | TCC | TLP, <i>Drosophila</i>             | ACQ <sup>a</sup> GDSGGP LVS | TCC |
| (IV) Esterases and lipases       |              |     | <i>Snake, Drosophila</i>           | HC <sup>a</sup> GDSGGP IHA  | TCC |
| Esterases                        | *            | Ser | V8, <i>Staph. aureus</i>           | TTG <sup>a</sup> NSGSPVFN   | TCA |
| ACE, <i>Drosophila</i>           | MTLFGESAGSS  | TCG | Prothrombin, human                 | ACE <sup>a</sup> GDSGGP FVM | AGT |
| ACE, <i>Torpedo</i>              | VTIFGESAGGA  | AGT | Factor IX, human                   | SCG <sup>a</sup> GDSGGP HVT | AGT |
| Esterase D, human                | MSIFGHSMGGH  | TCC | Factor X, human                    | ACQ <sup>a</sup> GDSGGP HVT | AGC |
| CU, <i>C. capsici</i>            | AVVAGYSQGT A | AGC | Protein C, human                   | ACE <sup>a</sup> GDSGGP MVA | AGT |
| CU, <i>C. gloeosporides</i>      | AIVSGYSQGT A | AGC | Plasminogen, bovine                | SCQ <sup>a</sup> GDSGGP LVC | AGC |
| TE, duck uropygial gland         | FALFGHSFGSF  | AGT | Plasminogen, human                 | SCQ <sup>a</sup> GDSGGP LVC | AGT |
| TE, rat mammary gland            | FAFFGHSGFSV  | AGT | CF C1r, human                      | ACQ <sup>a</sup> GDSGGV FAV | AGT |
| TE, rat FAS                      | YRVAGYSFGAC  | TCT | CF C1s, human                      | SCK <sup>a</sup> GDSGGV FAV | AGT |
| Lipases                          |              |     | TLP (hepsin), human                | ACQ <sup>a</sup> GDSGGP FVC | AGC |
| <i>Staphylococcus hyicus</i>     | VHFIGHSMGGQ  | AGT | apoLP, human                       | SCQ <sup>a</sup> GDSGGP LVC | AGT |
| Lingual, rat                     | IHYVGHSGGTT  | TCT | Related sequences                  | *                           |     |
| Hepatic, rat                     | VHLIGYSLGAH  | AGC | Haptoglobin, human                 | TCYGDAGSAFAV                | GCG |
| Lipoprotein, mouse               | VHLLGYSLGAH  | AGC | Protein Z, bovine                  | ARVWVAGGAVVR                | GCG |
| Lipoprotein, guinea pig          | VHLLGYSLGAH  | AGC |                                    |                             |     |

The active serine is starred and, in most cases, is that shown by experiment; otherwise it has been assigned by similarity of the sequence to the known active sequences. All the sequences are taken from the literature; a fully referenced and updated list may be obtained from the author. ACE, acetylcholinesterase; CU, cutinase; TE, thioesterase; LC, lecithin-cholesterol; FAS, fatty acid synthase; CF, complement factor; TPA, tissue plasminogen activator; UPA, urokinase plasminogen activator; CCP, cytotoxic T cell proteases; MCP, mast cell protease; PP, pancreatic protease; PAE, prostate arginine esterase; TLP, trypsin-like protease; apoLP, apolipoprotein. In, intron; <sup>a</sup>, present; †, absent; <sup>c</sup>, cDNA sequence; <sup>n</sup>, gene has no introns.

Table 1 also shows the relevant sequences for a number of proteins that lack the active serine and are devoid of catalytic function but have sequences similar to the corresponding enzymes from which they are likely to have been derived. We can now go one step further and suggest which of the serine enzymes might have been the precursor based on how codons are connected by single nucleotide mutations. Thus, haptoglobin and protein Z, with alanine, GCG, would have come from one of the trypsin class of enzymes with a TCN serine. It cannot be derived in one step from a AGN serine enzyme, nor from the presumed cysteine precursor. Similarly, thyroglobulin resembles cholinesterase<sup>8</sup>, and has arginine, CGT, which can be derived

from the AGN serine found in the vertebrate acetylcholinesterase. The vitellogenins are homologues of lipases<sup>9</sup>; they have asparagine and glycine and both could have been derived from a lipase with an AGN serine.

I have also asked whether there are instances of the presumed cysteine containing active sites which may still be used in modern proteins. Remarkably, GDCGG, the postulated predecessor of the serine protease sequence GDSGG, is thought to be the active site sequence of proteinase 2A of polio and related viruses<sup>10</sup>, while the sequence, GQCGG, has been suggested as the active site of poliovirus proteinase 3C<sup>11</sup>. The serine protease and esterase sites share the sequence GX SXG. The cysteine

homologue, GXCXG, as in GVCLG, for example, is found in several enzymes as the active site of a domain that transfers the amide group from glutamine<sup>12</sup>. Since these enzymes also have conserved sequences containing histidine they may be distant relatives of the esterases and proteases. GICRG is found at the active centre of the bacterial *luxA* gene product involved in the oxidation of long-chain fatty acid aldehydes<sup>13</sup> and may be related to the active site of modern lipases.

The demonstration that modern serine enzymes are likely to have arisen from cysteine precursors, encourages the search for evidence to connect the presumed and existing cysteine sequences with their postulated metallo-enzyme predecessors. Eventually, we may even hope to deduce the structures of the primitive peptides. Hypotheses can be tested by construction of the relevant sequence, either by genetic engineering methods or by peptide synthesis.

In serine-active enzymes there is a strong functional constraint on the serine to act as a nucleophile. In this stringent context, cysteine is the appropriate predecessor which connects the two serines. But, in other cases, threonine may be a more likely intermediate, and one instance is briefly noted here. A large family of proteins contain the sequence, GXXGXGKS, which is involved in the binding of a nucleoside triphosphate. Both codons for serine are found. For example, in the GTP-binding product of the mammalian gene, *N-ras*, the sequence is GAGGVGKS and the serine is AGC<sup>14</sup>; in the yeast gene, *RAS1*, with a closely similar sequence GGGGVGKS, the serine is TCT<sup>15</sup>. However, this family of ATP and GTP-binding domains frequently includes instances with GKT instead of GKS. Continuing with the example of GTP-binding proteins, we find the sequence GHVDSKGS in the protein synthesis elongation factor of yeast<sup>16</sup>, but GHVDHGKT in the *tufB* gene product of *E. coli*<sup>17</sup>. This suggests that threonine is an acceptable substitute for serine in these proteins, probably because the important constraint is the presence of a beta-hydroxyl group. Perhaps here threonine might be better called an intermediate rather than a predecessor; that is, the graph of codon connectivity is undirected.

The presence of both serine codons in sequences with invariant serines suggests multiple pathways of descent for that piece of DNA. The alternative of a single line of descent, with the two serines connected by double mutations or through missing sequences which have not been fixed, is dismissed by the strong correlation of codon representation with gene structure in the mammalian serine proteases. Gene structure is thus more important than sequence similarity for the construction of phylogenetic trees and suggests that multiple lines of descent might be realistically considered in other cases of sequence similarity but with differences in gene structure. The theory that everything was assembled from bits and pieces implies that there could have been many independent sources for the components in the course of evolution. There is even no difficulty in having two kinds of similar genes in the same genome, because if genes are composites, genomes are more so.

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## T cells can present antigens such as HIV gp120 targeted to their own surface molecules

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To trigger class II-restricted T cells, antigen presenting cells have to capture antigens, process them and display their fragments in association with class II molecules. In most species, activated T cells express class II molecules; however, no evidence has been found that these cells can present soluble antigens. This failure may be due to the inefficient capture, processing or display of antigens in a stimulatory form by T-cells. The capture of a soluble antigen, which is achieved by nonspecific mechanisms in macrophages and dendritic cells, can be up to  $10^3$  times more efficient in the presence of surface receptors, such as surface immunoglobulin on B cells that specifically bind antigen with high affinity<sup>1,2</sup>. We asked whether T cells would be able to present soluble antigens that bind to their own surface molecules. Here we show that such antigens can be effectively processed and presented by both CD4<sup>+</sup>- and CD8<sup>+</sup>-bearing human T cells. This indicates that T cells are fully capable of processing and displaying antigens and are mainly limited in antigen presentation by their inefficiency at antigen capture.

An antigen that can be targeted to the surface of any cell is an xenogenic antibody specific for a cell surface determinant. We have previously described human T cell clones that recognize mouse immunoglobulin in an MHC-restricted fashion<sup>3</sup>. Here we tested whether T cells could act as antigen presenting cells (APCs), and present a mouse monoclonal antibody (mAb) that binds to a T cell surface structure to such class II-restricted T cells. Clones of DR1<sup>+</sup> T cells bearing CD4 or CD8 were pulsed with different concentrations of mAbs that either do or do not bind to cell surface determinants and tested for their capacity to trigger proliferation of a DR1-restricted T-cell clone specific for processed mouse  $\kappa$  light chains.

Figure 1a shows that both CD4<sup>+</sup> and CD8<sup>+</sup> T cells are effective in presenting the mouse mAb OKT9 which binds to the transferin receptor, but fail to present a different mAb, DA4-4, which is specific for human IgM and does not specifically bind to the cell surface. Figure 1b shows that anti-CD4 and anti-CD8 mAbs could be presented only by CD4<sup>+</sup> or by CD8<sup>+</sup> T cells respectively, indicating again that activated T cells can present the soluble antigen 'mouse immunoglobulin' only if this binds to a cell surface molecule. In addition, although antibodies that do not bind to the cell surface could be presented at high concentrations by Epstein-Barr virus-transformed B cells (EBV-B cells) or macrophages<sup>3</sup>, we could never demonstrate presentation by T cells, even when these antibodies were used at concentrations as high as  $100 \mu\text{g ml}^{-1}$ .

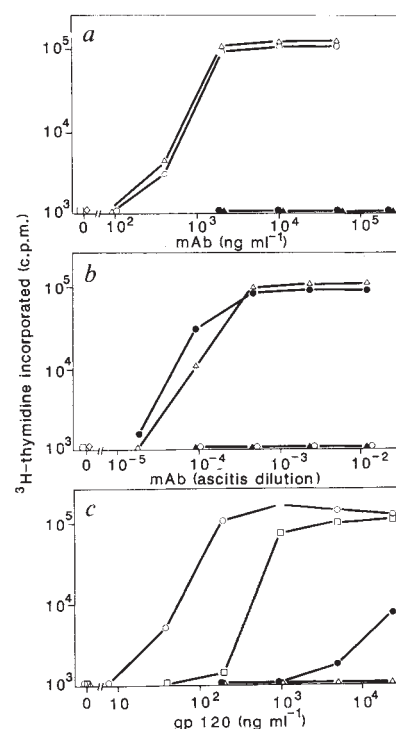
It could be argued that antibodies are somewhat artificial antigens. We therefore looked for a naturally occurring antigen that could bind selectively to T cells. The human immunodeficiency virus envelope glycoprotein (HIV-gp120) binds with high affinity ( $\sim 10^{-9}$ ) to CD4 present on a subset of T cells and on macrophages<sup>4</sup>. We asked whether the binding to T cells would facilitate the capture of gp120 and would subsequently allow it to be processed and presented to class II-restricted T cells.

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**Fig. 1** T cells can present antigens that bind to their surface structures. *a*, Proliferative response of a DR1-restricted T cell clone specific for processed mouse  $\kappa$  chains in the presence of autologous APCs. The APCs were: a CD4<sup>+</sup> clone pulsed with different concentrations of OKT9 ( $\Delta$ ), DA4-4 ( $\blacktriangle$ ) or medium alone ( $\square$ ); a CD8<sup>+</sup> clone pulsed with OKT9 ( $\circ$ ), DA4-4 ( $\bullet$ ) or medium alone ( $\diamond$ ). *b*, Proliferative response of the same T cell clone as in panel *a*. APCs were: a CD4<sup>+</sup> clone pulsed with OKT4A ( $\Delta$ ), OKT8 ( $\blacktriangle$ ) or medium alone ( $\square$ ); a CD8<sup>+</sup> clone pulsed with OKT4A ( $\circ$ ), OKT8 ( $\bullet$ ) or medium alone ( $\diamond$ ). *c*, Proliferative response of a DR1-restricted T cell clone specific for gp120 cultured in the presence of autologous APCs that were pulsed with different concentrations of gp120. The APCs were: a CD4<sup>+</sup> T cell clone ( $\circ$ ); a CD8<sup>+</sup> T cell clone ( $\Delta$ ); an EBV-B cell ( $\square$ ); a CD4<sup>+</sup> T cell clone that had been prepulsed with OKT4A mAb ( $\bullet$ ).

**Methods. Reagents:** Recombinant HIV-gp120 was produced and purified as described<sup>12</sup>. OKT4A (anti CD4) was kindly provided by Dr Ellis Reinherz (Dana Farber Cancer Institute). The OKT9 (anti-transferrin receptor), OKT8 (anti CD8) and DA4-4 (anti-human IgM) hybridomas were obtained from the American Type Culture Collection. **T cell clones:** The conditions for isolation and maintenance of human T-cell clones have been described elsewhere<sup>2,3</sup>. A DR1-restricted T-cell clone specific for processed mouse  $\kappa$  chain determinants has already been described<sup>3</sup>. In order to isolate gp120-specific T-cell clones, PBMCs from 5 healthy seronegative donors were cultured at  $8 \times 10^5$  cells ml<sup>-1</sup> in RPMI-5% human serum with  $1 \mu\text{g ml}^{-1}$  recombinant gp120 in 200  $\mu\text{l}$  cultures in flat bottom Costar microplates. After 7 days IL-2 (Roche) was added at 30 U ml<sup>-1</sup> and, after an additional 5 days, cultures containing growing cells were chosen by visual inspection. The growing cells were expanded in IL-2, washed and tested for their capacity to proliferate in response to gp120 in the presence of autologous irradiated PBMCs. The cultures showing gp120 specific proliferation were cloned by limiting dilution. From the same donors we also isolated random T-cell clones by directly cloning PBMCs with phytohaemagglutinin (Wellcome). CD4<sup>+</sup> and CD8<sup>+</sup>, CD4<sup>-</sup> clones were selected and used as APCs. To exclude the presence of contaminating APCs from the irradiated PBMCs these T cell clones were isolated and restimulated using as feeders allogeneic PBMCs that lacked the appropriate class II molecules. We also isolated EBV-B cells from these donors according to a standard procedure<sup>2</sup>. **Proliferation assay:** T-cell clones or EBV-B cells were pulsed with different concentrations of gp120 or mAbs for 4 h at 37 °C, washed 4 times, irradiated (3,000R) and tested for their capacity to trigger proliferation of antigen-specific T-cell clones. To block the uptake of gp120 the OKT4A mAb was added (1:500 dilution of ascites) at 37 °C 1 h before the pulsing with gp120.  $2 \times 10^4$  responder T cells were cultured with irradiated APCs ( $2 \times 10^4$  EBV cells or  $10^5$  T cells) in 200  $\mu\text{l}$  RPMI 10% fetal calf serum in 96-well flat bottom microplates. After 2 days  $1 \mu\text{Ci}$  of  $^3\text{H}$ -thymidine (Amersham, specific activity 5 Ci mmol<sup>-1</sup>) was added and the radioactivity incorporated was measured after an additional 16 h by liquid scintillation counting.



Class II-restricted T-cell clones, specific for gp120, were isolated from five seronegative healthy donors by stimulating *in vitro* peripheral blood mononuclear cells (PBMCs) with recombinant gp120. All these clones are CD3<sup>+</sup>, WT31<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>-</sup> and proliferate in response to autologous PBMCs or EBV-B cells that have been pulsed with gp120. Their response to gp120 is MHC-restricted in that it can be blocked by anti-class II antibodies and maps to DR1, DR2 or DR5 in different clones (data not shown). For APCs we isolated random CD4<sup>+</sup>, CD8<sup>-</sup> and CD8<sup>+</sup>, CD4<sup>-</sup> T cell clones as well as EBV-B cells from the same donors and compared their capacity to present gp120. In

these experiments the APCs were pulsed at 37 °C for 4 h with different concentrations of gp120 and tested for their capacity to trigger proliferation of the gp120-specific T-cell clones.

Figure 1c shows that CD4<sup>+</sup> T cells pulsed with concentrations of gp120 as low as 40 ng ml<sup>-1</sup>, were indeed stimulatory for gp120-specific T cells, whereas CD8<sup>+</sup> T cells were not stimulatory, even when pulsed with concentrations of gp120 as high as 25  $\mu\text{g ml}^{-1}$ . In addition, CD4<sup>+</sup> T cells that had been pretreated with the mAb OKT4A, which is known to prevent binding of gp120<sup>5,6</sup>, were not able to trigger T cells. Figure 1c also shows that EBV-B cells can present gp120, but less effectively than CD4<sup>+</sup> T cells. Although it is known that EBV-B cells are capable of taking up antigens nonspecifically, it should be noted that the EBV-B cells used in these experiments express low levels of CD4 that might well enhance their capacity to capture gp120.

To attempt to determine whether gp120 must be processed by the T cells before being presented, we used an assay in which we measured the capacity of gp120-pulsed T cells to induce  $\text{Ca}^{2+}$  mobilization in gp120-specific T-cell clones. Table 1 shows that CD4<sup>+</sup> T cells that had been pulsed in the cold with gp120 and warmed to 37 °C for up to 45 min are not capable of inducing  $\text{Ca}^{2+}$  mobilization in gp120-specific T cells. However, these cells in time acquire the capacity to trigger gp120-specific T cells and after 90 min at 37 °C they become fully stimulatory. This capacity is not acquired if the T cells are incubated at 37 °C in the presence of the protease inhibitor, leupeptin. The presentation of mAbs by B cells<sup>3</sup> and by activated T cells (data not shown) also requires processing. These results indicate that gp120, like conventional antigens, must be processed and that T cells, like conventional APCs, are capable of performing this job.

We can therefore conclude from the above experiments that activated class II positive T cells can process and present antigens, such as gp120 and mouse immunoglobulin, but only if these antigens bind to cell surface receptors, suggesting that the antigen presenting capacity of T cells is limited only by their inefficiency in capturing antigens and not, as previously postu-

**Table 1** Processing of gp120 by CD4<sup>+</sup> T cells

| APC          |                    | % Of responding cells | [Ca <sup>2+</sup> ] (nM) |
|--------------|--------------------|-----------------------|--------------------------|
| T cells      | Time at 37 °C      |                       |                          |
| unpulsed     | 0 min              | <3                    | 166                      |
| gp120-pulsed | 15 min             | <3                    |                          |
| gp120-pulsed | 45 min             | <3                    |                          |
| gp120-pulsed | 90 min             | 56                    | 550                      |
| gp120-pulsed | 300 min            | 44                    | 1,200                    |
| gp120-pulsed | 20 h               | 77                    | 1,400                    |
| gp120-pulsed | 90 min + leupeptin | <3                    |                          |

A CD4<sup>+</sup> T cell clone was pulsed with  $2 \mu\text{g ml}^{-1}$  gp120 on ice, washed and tested after different times of incubation at 37 °C for its capacity to induce  $\text{Ca}^{2+}$  mobilization in an Indo-1-loaded gp120-specific T-cell clone. Some cells were pulsed and incubated in the presence of leupeptin (Sigma) at  $5 \mu\text{g ml}^{-1}$ . gp120-specific T cells were loaded with Indo-1-AM (Calbiochem) as described<sup>13</sup>. To test whether the gp120-pulsed cells had acquired the capacity to activate a gp120-specific T cell clone, the responder T cells ( $10^5$ ) were pelleted with the antigen presenting T cells ( $10^6$ ) and incubated at 37 °C. After 5 min, the cells were resuspended and the ratio of Indo-1 low wavelength (405 nm) to high wavelength (485 nm) was measured in single cells using a FACS440 equipped with a argon laser (354 nm, 50 mW). Calibration of this ratio as a function of  $\text{Ca}^{2+}$ -concentration was carried out as described<sup>14</sup>.

lated, by defects in the processing machinery<sup>7</sup> or by the delivery of inhibitory signals<sup>8,9</sup>. *In vivo*, the antigen presenting function of T cells will generally be insignificant because very few proteins will bind directly to T cells. However, there are instances, for example the case of gp120, in which the situation might change dramatically. The fact that gp120 can bind to CD4<sup>+</sup> cells and be selectively presented could therefore have immunopathological consequences for HIV-1 infection. Because gp120 is readily shed from the surface of HIV-1 infected cells<sup>10,11</sup>, the possibility exists that free gp120 might bind to uninfected CD4<sup>+</sup> T cells and macrophages and target them for destruction by gp120-specific cells. We are currently testing this possibility.

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## Identification of a protein encoded by the *vpu* gene of HIV-1

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Human immunodeficiency virus 1 (HIV-1) is the aetiological agent of AIDS<sup>1-3</sup>. The virus establishes lytic, latent and non-cytopathic productive infection in cells in culture<sup>4,5</sup>. The complexity of virus-host cell interaction is reflected in the complex organization of the viral genome<sup>6-9</sup>. In addition to the genes that encode the virion capsid and envelope proteins and the enzymes required for proviral synthesis and integration common to all retroviruses, HIV-1 is known to encode at least four additional proteins that regulate virus replication, the *tat*, *art*, *sor* and 3' *orf* proteins, as well as a protein of unknown function from the open reading frame called R<sup>10-18</sup>. Close examination of the nucleic acid sequences of the genomes of multiple HIV isolates raised the possibility that the virus encodes a previously undetected additional protein. Here we report that HIV-1 encodes a ninth protein and that antibodies to this protein are detected in the sera of people infected with HIV-1. This protein distinguishes HIV-1 isolates from the other human and simian immunodeficiency viruses (HIV-2 and SIV)<sup>19-21</sup> that do not have the capacity to encode a similar protein.

Figure 1a is a schematic diagram of the open reading frames of the region between the first coding exons of the *tat* and *art* genes of HIV-1 and the envelope glycoprotein gene. In this

region many strains of the virus have the capacity to encode a protein of 80-82 amino acids that initiates with an AUG codon (Fig. 1b). To examine this possibility, two oligopeptides were made that correspond in sequence to regions of the protein which were predicted, on the basis of amino acid sequence, to be hydrophilic. One corresponded to amino acids 29 to 41 (peptide 1), and the other to amino acids 73 to 81 (peptide 2) (Fig. 1b). The amino acid sequences corresponded to the protein that BH10 substrain of the IIIB isolate was predicted to make<sup>6</sup>. The peptides were conjugated to keyhole limpet haemocyanin and used to raise antibody in three rabbits each. After multiple injections of the antigen, the rabbits were shown to produce antibodies that recognized the oligopeptide (data not shown).

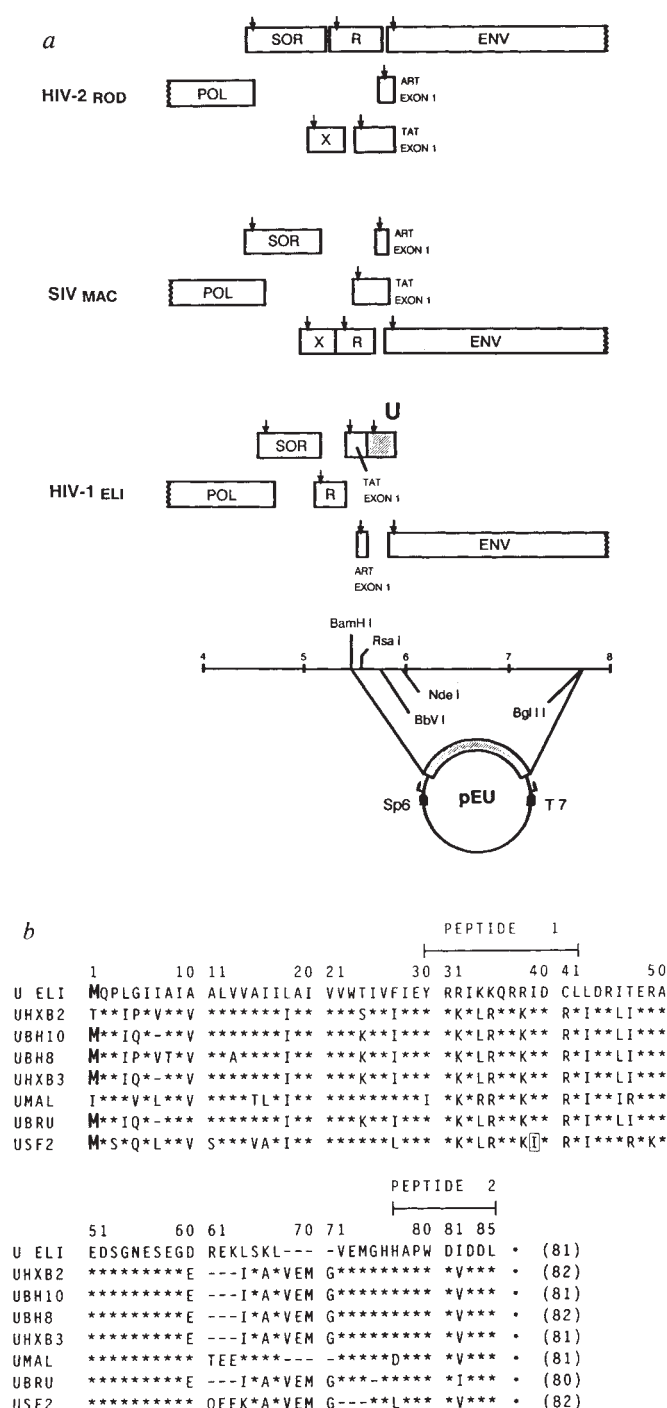
The ability of the region between the first coding exon of *tat* and the *env* gene to encode a protein was first examined by an *in vitro* translation assay in a reticulocyte lysate<sup>22</sup>, using RNA made *in vitro*<sup>23</sup>. RNA was made from a restriction fragment, 2,231 nucleotides long, of an HIV provirus that spanned the region between the first coding exons of the *tat*, *art* and part of the *env* genes. The template was derived from a fragment of the provirus of the ELI strain of HIV-1 placed 3' to the SP6 bacteriophage RNA polymerase promoter<sup>23</sup> (Fig. 1a). This strain was selected as it contains an open reading frame in this region that initiates with an AUG codon (Fig. 1b)<sup>24</sup>. The viral sequences present in this RNA transcript, as shown in Fig. 1a, extend from the 5' end of the first coding exon of the *tat* (*Bam*HI site) to 1,839 nucleotides (*Bgl*II site) within the *env* sequence. However, the initiation codon for the *tat* gene is not intact in this RNA as *Bam*HI cleaves the ELI proviral strain between the T and G residues of the *tat* initiation codon.

Proteins produced in the *in vitro* lysate using the RNA derived from this proviral fragment were labelled with <sup>35</sup>S-methionine and separated by size using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2a). The proteins synthesized in this system are displayed in lane 2. The proteins precipitated by rabbit anti-peptide-2 serum are also shown. Two proteins of relative molecular mass of approximately 15,000 (15K) and 16,000 (16K) are evident in the unfractionated extract and are precipitated by the rabbit antisera. The 15K and 16K proteins are not precipitated by the pre-immune rabbit sera (lane 3). All three of the antisera to peptide 2 recognize both proteins (lane 4) as do the antisera to peptide 1, albeit more weakly (data not shown). The data of Fig. 2a also show that peptide 2 competes for recognition of the 15K and 16K proteins by antisera (lane 5). However, peptide 1 (lane 6) or an unrelated peptide do not compete with anti-peptide-2 serum (lane 7).

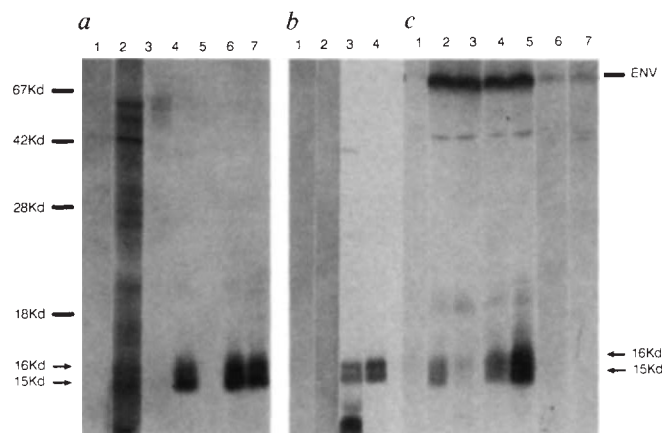
To confirm the origin of the proteins, RNA from other proviral fragments was used in the *in vitro* translation assay. In one set of experiments, the template used for synthesis of RNA was truncated by restriction enzyme cleavage either seven nucleotides 5' to the proposed AUG codon (*Rsa*I site) or 30 nucleotides 3' to the proposed AUG codon (*Bbv*I site) (Fig. 1a). No specific protein products recognized by anti-peptide-2 antiserum were observed in these experiments (Fig. 2b, lanes 1 and 2). When the template used for synthesis of RNA was cleaved 102 nucleotides 3' to the proposed stop codon (*Nde*I site), the 15K and 16K proteins were detected using anti-peptide-2 serum (Fig. 2b, lanes 3 and 4).

To examine the possibility that the proteins corresponding to the 15K and 16K products are produced in natural infections, the ability of antisera from normal and AIDS patients to recognize the protein synthesized *in vitro* was tested. The data of Fig. 2c demonstrate that HIV seropositive patient antisera recognize both the 15K and 16K proteins (lanes 2, 4 and 5). The ability of antiserum to precipitate the two proteins is partially competed out by peptide 2 (lane 3). The 15K and 16K proteins are not recognized by normal human serum (lane 1). However, all of the 19 sera of HIV-1 infected patients that immunoprecipitated the truncated *env* product were found to precipitate both the





**Fig. 1** *a*, Genetic organization of the central region of HIV-1 (ELI isolate, ref. 24) compared with SIV<sup>20,21</sup> and HIV-2 (ROD isolate, ref. 19). Arrows indicate the initiator AUG codons in viral genes. SP6 plasmid used to synthesize messenger RNA, a *Bam*HI to *Bgl*II fragment, 2,231 nucleotides long, from the HIV ELI provirus that spanned the region between the first coding exons of the *tat*, *art* and part of the *env* gene was cloned 3' to the SP6 bacteriophage RNA polymerase promoter<sup>23</sup>. Internal restriction sites used to linearize the plasmids are indicated. *b*, Alignment of the *vpu* gene protein sequence. The ELI isolate is taken as reference. Gaps (—) were introduced to optimize the alignment. Asterisks indicate amino acid identity. The HIV isolates compared include ELI, MAL (ref. 24), HXBc2, BH-10, BH-8, pHXB3 (ref. 6), BRU (ref. 7) and USF2 (ref. 8). USF2 contains a termination codon at position 39 ([I]). However, a -1 frameshift results in an extension of 43 amino acids that are well conserved when compared with the ELI U sequence.

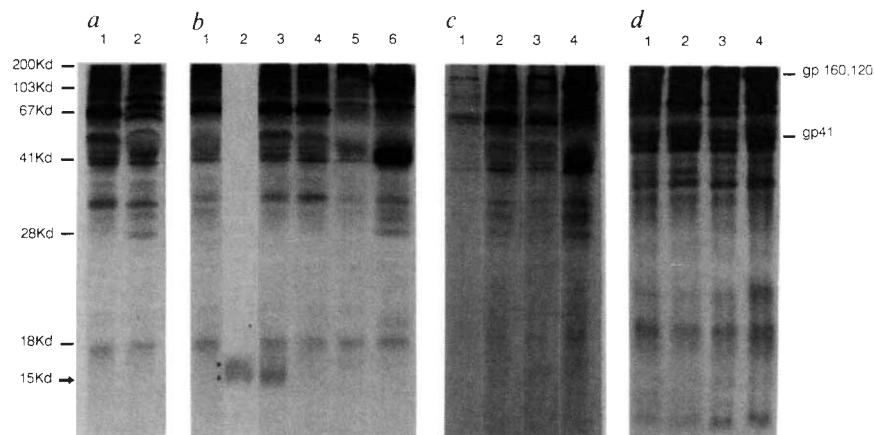


**Fig. 2** *In vitro* characterization of the *vpu* gene product. *a*, pEU plasmid was linearized by digestion at an *Eco*RI site located in the polylinker 3' to the HIV<sub>ELI</sub> insert and used as template for *in vitro* transcription by SP6 RNA polymerase as described<sup>26</sup> except that the concentration of GTP and cap analogue m<sup>7</sup>GpppG were raised to 0.2 and 1.0 mM respectively. Messenger RNAs were labelled with [5'-<sup>3</sup>H] CTP and purified as described<sup>26</sup>. *In vitro* translation of equimolar amounts of RNA (equal amounts of radioactivity) was performed in reticulocyte lysate<sup>22</sup>. Incubation was at 30 °C for 30 min in the presence of <sup>35</sup>S-methionine. Labelled products were analysed directly by 15% SDS-PAGE (lane 2) or immunoprecipitated<sup>27</sup> beforehand with pre-immune rabbit serum (lane 3); anti-peptide-2 serum (lane 4); anti-peptide-2 serum in the presence of 500 µM of peptide 2 (lane 5), peptide 1 (lane 6), or an unrelated peptide QEAEATATKSSC (lane 7). Lane 1 represent a total translation reaction with no mRNA added. *b*, pEU plasmid was linearized with the following restriction enzymes *Rsa*I (lane 1); *Bbv*I (lane 2) and *Nde*I (lanes 3 and 4). SP6-generated RNAs were translated *in vitro* and immunoprecipitation was performed on the labelled products using anti-peptide-2 serum (lanes 1, 2 and 4). Lane 3 represents a total *in vitro* translation reaction. *c*, After *in vitro* translation of SP6-generated pEU RNA, the labelled products were immunoprecipitated as described<sup>27</sup> except that 1 M NaCl was used in the immunoprecipitation reaction. Immunoprecipitation with a pool of normal human serum (lane 1); HIV-1-infected human sera (lanes 2, 4 and 5); HIV-1-infected patient serum in the presence of 500 µM of peptide 2 (lane 3); HIV-2-infected human serum (lane 6) or SIV-infected *Rhesus macaques* serum (lane 7). Fifteen HIV-2-infected human serum and four SIV-infected *Rhesus macaques* serum were tested. These sera were demonstrated to specifically react with HIV-2 or SIV proteins by immunoprecipitation and Western blot analysis (not shown). None of these antisera immunoprecipitated p15<sup>vpu</sup> and p16<sup>vpu</sup>. Immunoprecipitates were resolved on 15% SDS-PAGE.

15K and 16K proteins (data not shown). Antisera from HIV-2-infected humans or from SIV-infected macaques do not precipitate either protein (Fig. 2c, lanes 6 and 7).

We examined whether the anti-peptide-2 serum recognized the 15K and 16K proteins in three cell lines that constitutively express HIV-1 proteins *art* and *env* encoded by the 3' half of the virus. Cloned HeLa cell lines that have stably integrated the region between the *art* gene and the 3' long terminal repeat (LTR) of the proviral ELI, HXBc2 and MAL strains<sup>24,6</sup> of HIV were isolated (Terwilliger *et al*, in preparation). The plasmids used for construction of these cell lines contained the HIV LTR juxtaposed 5' to the initiation codon of the *art* gene. The *tat* gene product was supplied *in trans*. Figure 3b shows that the anti-peptide-2 antiserum specifically recognized a 15K protein in the cell line derived from the ELI provirus (lane 3) that comigrates with the 15K protein made *in vitro* (lane 2). The same antiserum does not recognize a protein in the cell line that expresses proteins derived from the MAL (Fig. 3c) or the HXBc2

**Fig. 3** Identification of the *vpu* gene product in cell lines constitutively expressing proteins encoded by the 3' half of the HIV-1 ELI provirus. HeLa cell lines that have the region of the HIV-1 provirus between the *art* gene and the 3' LTR of the HIV proviral ELI, HXBc2 and MAL strains stably integrated were constructed (Terwilliger *et al.*, in preparation). The parental cells used to isolate these cell lines had previously been selected for constitutive expression of the *tat* gene product, following infection with a retroviral vector carrying the *tat* coding sequences<sup>28</sup>. Cells were labelled with <sup>35</sup>S-methionine and cysteine and cell lysates were immunoprecipitated as described<sup>27</sup>. *a*, HeLa *tat* cell line lysates immunoprecipitated with anti-peptide-2 serum (lane 1) or HIV-1-infected patient serum (lane 2). *b*, HeLa *tat* ELI lysates immunoprecipitated with pre-immune rabbit serum (lane 1); anti-peptide-2 serum (lane 3); anti-peptide-2 serum in the presence of 500  $\mu$ M of peptide 2 (lane 4); normal human serum (lane 5); HIV-1-infected patient serum (lane 6). Lane 2 represent an immunoprecipitation of labelled *in vitro* translated product from pEU RNA with anti-peptide-2 serum. *c*, HeLa *tat* MAL lysate immunoprecipitated with pre-immune rabbit serum (lane 1); anti-peptide-2 serum (lane 2); normal human serum (lane 3) and HIV-1-infected patient serum (lane 4). *d*, HeLa *tat* IIIB lysate immunoprecipitated with pre-immune rabbit serum (lane 1); anti-peptide-2 serum (lane 2) normal human serum (lane 3) and HIV-1-infected patient serum (lane 4).



(Fig. 3d) proviruses. This result was expected as neither of the proviruses contain a properly positioned initiation codon at the 5' end of the open reading frame (Fig. 1b). The absence of detection of the 15K protein by the HIV-1 patient antiserum in the cell line derived from the ELI provirus is probably due to both the low antibody titre in the antiserum used and the much smaller amount of the 15K protein in the cell line compared to the *in vitro* translation products.

The experiments presented here demonstrate that HIV-1 has the capacity to encode a previously unrecognized protein. The open reading frame from which this protein is synthesized was originally designated U (ref. 7) and so we propose to call the gene *vpu*, for viral protein U, and the proteins produced p15<sup>vpu</sup> and p16<sup>vpu</sup>. The product of *vpu* is made upon HIV-1 infection as antisera from the majority of HIV-1-infected people surveyed have antibodies that recognize the protein.

All HIV-1 proviral strains isolated contain an open reading frame in the region corresponding to *vpu*. However, the ability of the individual proviral strains to produce a protein from this region is compromised in some strains by a single point mutation that prevents *vpu* expression. Indeed, different proviral strains from the same viral isolate differ in their ability to encode *vpu*: independent proviral clones of the IIIB isolate, HXBc2, BH10, BH-8 and BH-3 are an example (Fig. 1b). There is a similar variation in the ability of individual proviral clones to encode other viral proteins, for example, the 3' *orf* product. The mutation that truncates the protein product of the IIIB 3' *orf* yields a virus that replicates more rapidly in culture than the wild-type virus<sup>15</sup>. The virus produced by transfection with HXBc2 can grow in T cells in culture<sup>25</sup> implying that a virus which cannot express *vpu* can replicate. However, the ability of the *vpu*<sup>-</sup> virus to replicate does not rule out the possibility that the *vpu* product is important in regulation of viral replication or pathogenesis.

The *vpu* gene distinguishes HIV-1 from HIV-2 and SIV infections. A computer-assisted search for proteins similar to p15/16<sup>vpu</sup> showed that HIV-2 and SIV do not encode a similar protein. HIV-2 and SIV strains do contain an open reading frame that is missing from that of HIV-1 isolates, the X open reading frame<sup>19</sup>, but there is no predictable similarity in the predicted protein products of *vpu* and the X open reading frame. None of the sera of HIV-2-infected patients surveyed contained antibodies to the *vpu* product, nor were antibodies to *vpu* detected in Rhesus macaques infected with SIV.

We note that *vpu* is highly conserved amongst the HIV-1 proviral sequences isolated (Fig. 1b), and that *vpu* is removed by splicing from viral messenger RNAs that encode regulatory proteins<sup>9,11,16</sup>. It is therefore predicted that the *vpu* product is not made in the absence of the *art* gene product as only fully spliced messenger RNAs accumulate in the absence of this product<sup>16,17</sup>. We suspect that the *vpu* product is made late in infection like virion proteins.

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# Transcriptional activation of *c-jun* during the $G_0/G_1$ transition in mouse fibroblasts

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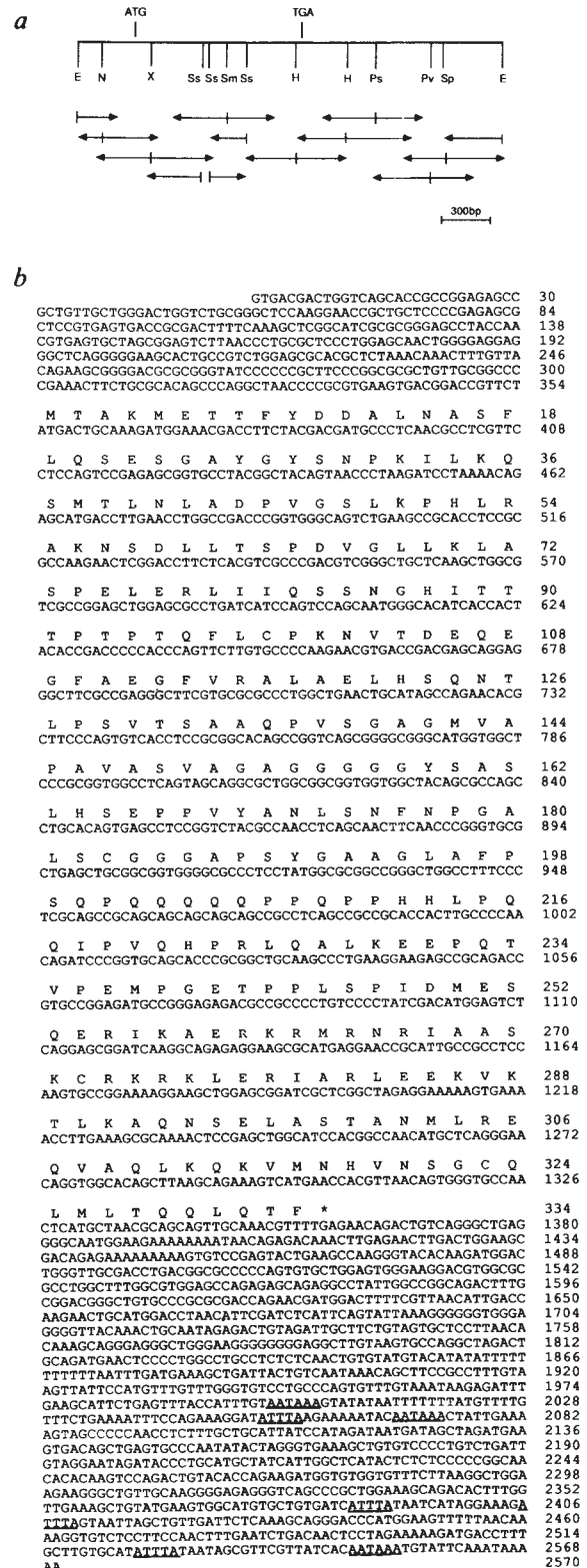
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Before quiescent cells can respond to mitogens and progress through the  $G_1$  phase of cell growth, new messenger RNA synthesis is required<sup>1</sup>. The  $G_1$  phase seems to be a critical point of control in the cell cycle, where normal cells deprived of growth factors halt cycling while transformed cells do not, suggesting that regulatory genes, uncontrolled in the neoplastic phenotype, are expressed during the  $G_0$  to  $G_1$  transition. Some of these may code for nuclear proteins that participate in the transactivation of genes required for the progression through  $G_1$ . The observed changes in expression of the proto-oncogenes *c-fos* and *c-myc*, following stimulation of fibroblasts with growth factors<sup>2-7</sup>, support this notion as recent evidence suggests that c-FOS and c-MYC proteins can function as transactivating factors<sup>8-12</sup>. Moreover, the rapid induction of several genes in fibroblasts coding for putative transacting factors during the  $G_0$  to  $G_1$  transition has been recently reported<sup>13-16</sup>. Here we present the nucleotide sequence of a mouse cDNA clone coding for a 334 residue protein which shows 80% similarity with v-JUN<sup>17</sup> and more than 98% similarity with the human c-JUN sequence<sup>18,19</sup>. We have demonstrated that in quiescent fibroblasts *c-jun* transcription is rapidly induced during the  $G_0$  to  $G_1$  transition.

We have previously described the isolation of over 70 non-overlapping sequences by differential screening of a  $\lambda$  cDNA library prepared with RNA from quiescent cells treated with serum for 4 hours in the presence or cycloheximide<sup>20</sup>. These cDNAs should correspond to genes activated during the  $G_0/G_1$  transition as a direct consequence of the signals triggered by the binding of growth factors to their receptors. To determine if genes related to v-jun<sup>17</sup> were present among the isolated sequences, the cDNAs were probed with a synthetic oligonucleotide corresponding to a segment of the DNA binding domain of v-jun. One single clone (AH119) with an insert of 2.6 kilobase (kb) gave a strong signal on hybridization with this probe, even under stringent conditions (not shown). The mRNA corresponding to AH119 (2.7–3.2 kb) proved to be undetectable in quiescent cells but is serum-inducible, even in the presence of cycloheximide (see below).

The complete nucleotide sequence and predicted amino acid sequence of AH119 are shown in Fig. 1. The sequence is 2,565 nucleotides long with a 5' flanking sequence of 354 nucleotides, an open reading frame of 1,002 nucleotides and a long 3' untranslated region of 1,209 nucleotides. The 3' end terminates with a short stretch of A residues located 11 nucleotides downstream from an AATAAA polyadenylation signal consensus sequence<sup>21</sup>. The open reading frame extends from nucleotides 355 to 1,356 and codes for a polypeptide 334 amino acids long with a relative molecular mass of 35,941. From the estimated size of AH119 mRNA (2.7–3.2 kb) the complete 5' flanking sequence could be as long as 950 nucleotides. The 3' untranslated region contains four copies of the sequence ATTTA which is potentially involved in mRNA selective degradation<sup>22</sup>, and two additional AATAAA consensus polyadenylation signals (underlined in Fig. 1b) at nucleotide positions 1,961–1,966 and 2,069–2,074.

The deduced amino acid sequence of AH119 is very similar to that of the v-JUN protein, with the exception of three regions that are absent in the viral protein. As a result v-JUN protein (without considering the gap portion) is 38 amino acids shorter



**Fig. 1** Nucleotide sequence of AH119 cDNA and predicted amino acid sequence of the protein. *a*, Restriction map and sequencing strategy. *b*, Nucleotide sequence with consensus signal for poly(A) addition and ATTTA sequences potentially involved in mRNA degradation underlined. E, *EcoRI*; H, *HpaI*; N, *NheI*; Ps, *PstI*; Pv, *PvuII*; Sm, *SmaI*; Ss, *SstI*; Sp, *SphI*; X, *XhoI*. **Methods.** The original clone was digested with various restriction enzymes and the fragments cloned in M13-derived vectors. Single-stranded DNA was obtained<sup>26</sup> and sequenced by the dideoxynucleotide procedure<sup>27</sup>. UWGCG programs were used for nucleotide and amino acid sequence analysis.

**Fig. 2** Amino acid sequence comparison of AH119 with human *c-jun*. The human *c-jun* sequence was taken from ref. 19.

|     |                                                    |     |       |
|-----|----------------------------------------------------|-----|-------|
| 1   | MTAKMETTFYDDALNASFLQSESGAYGYSNPKILKQSMNTLNLDVPVGLK | 50  | AH119 |
| 1   | MTAKMETTFYDDALNASFLQSESGPYGYSNPKILKQSMNTLNLDVPVGLK | 50  | CJUN  |
| 51  | PHLRKNSDLLTSPDVGLLKLASPELERLIQSSNGHITTTPTQFLCP     | 100 | AH119 |
| 51  | PHLRKNSDLLTSPDVGLLKLASPELERLIQSSNGHITTTPTQFLCP     | 100 | CJUN  |
| 101 | KNVTDEQEGFAEGFVRALAEHLSQNTLPSTAAQPVSGAGMVAVASV     | 150 | AH119 |
| 101 | KNVTDEQEGFAEGFVRALAEHLSQNTLPSTAAQPVNGAGMVAVASV     | 150 | CJUN  |
| 151 | AGAGGGGGYSASLHSEPPVYANLSNPNPGLSCGGGAPSYGAAGLAFPSQ  | 200 | AH119 |
| 151 | AGGSGGGGYSASLHSEPPVYANLSNPNPGLSSGGGAPSYGAAGLAFPAQ  | 200 | CJUN  |
| 201 | PQQQQPPQPPHLLPQQIPVQHPRLQALKEEPTVPEMPGETPPLSPIDM   | 250 | AH119 |
| 201 | PQQQQ...QPPHLLPQQMPVQHPRLQALKEEPTVPEMPGETPPLSPIDM  | 247 | CJUN  |
| 251 | ESQERIKAERKMRNRRIAASKCRKKRLERLARLEEKVKTILKAQNSLAST | 300 | AH119 |
| 248 | ESQERIKAERKMRNRRIAASKCRKKRLERLARLEEKVKTILKAQNSLAST | 297 | CJUN  |
| 301 | ANMLREQVAQLKQKVMNHVNSGCQLMLTQQLQTF                 | 334 | AH119 |
| 298 | ANMLREQVAQLKQKVMNHVNSGCQLMLTQQLQTF                 | 331 | CJUN  |

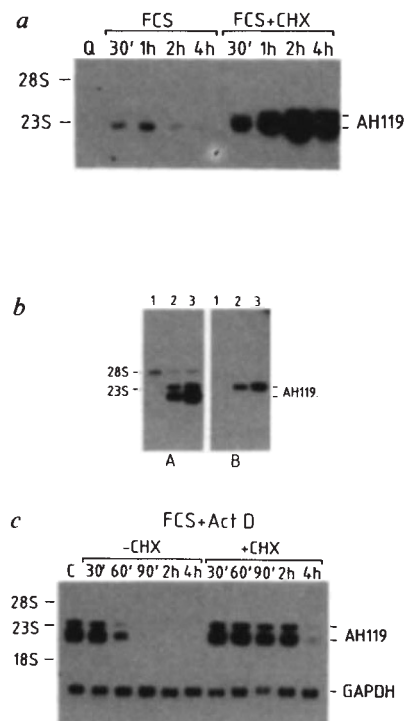
**Fig. 3** *a*, Northern blot analysis of mRNA from quiescent cells treated with serum for the indicated times in the absence (FCS) or presence (FCS + CHX) of cycloheximide. The complete AH119 cDNA was used as a probe. *b*, Poly(A)<sup>+</sup> RNA samples (0.2 µg) from quiescent cells (1) and cells treated with cycloheximide for 4 h in the absence (2) or presence (3) of serum were fractionated in agarose-formaldehyde gels, transferred to nitrocellulose filters, and hybridized against complete AH119(A) or a probe comprising nucleotides 2,130–2,570 (B). *c*, RNA from quiescent cells stimulated with 20% FCS in the presence of cycloheximide for 4 h (C) followed by incubation in FCS plus actinomycin D in the absence (–CHX) or presence (+CHX) of cycloheximide for different times. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as control.

**Methods.** NIH 3T3 cells were routinely grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and antibiotics (100 units ml<sup>-1</sup> penicillin, 50 µg ml<sup>-1</sup> streptomycin). Confluent cells were made quiescent by incubation for 48 h in medium containing 1% FCS. Cultures were stimulated by refeeding with medium containing 20% FCS. When used, cycloheximide was added at 10 µg ml<sup>-1</sup> and actinomycin D at 1 µg ml<sup>-1</sup>. Total RNA was prepared from cells using the guanidine hydrochloride procedure<sup>28</sup>. Poly(A)<sup>+</sup> RNA was isolated by oligo(dT) cellulose (Collaborative Research) column chromatography<sup>29</sup>. The RNA was separated on agarose gels containing 6% formaldehyde<sup>30</sup> and blotted on to Gene Screen Plus (New England Nuclear). Purified inserts<sup>31</sup> were <sup>32</sup>P-labelled by nick translation to a specific activity of 1–5 × 10<sup>8</sup> c.p.m. µg<sup>-1</sup> (ref. 32). Hybridization was carried out in 50% formamide, 0.5% SDS, 5 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate), and 5 × Denhardt's solution at 42 °C for 40 h. Filters were extensively washed in 0.1 × SSC containing 0.5% SDS at 56 °C.

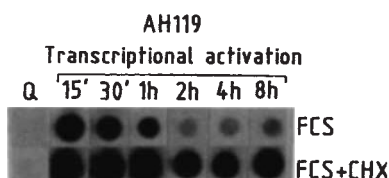
than the predicted product of AH119. The overall similarity of the two proteins is 80%, but 98% similarity is found in the region where the putative DNA binding domain is located (not shown). Confirmation that AH119 corresponds to mouse *c-jun* comes from comparison with the nucleotide and predicted amino acid sequence of human *c-jun*<sup>19</sup>. The overall similarity between these two proteins is 98%, and is 100% in the last 119 amino acids (Fig. 2). Comparison of their nucleotide sequences revealed an overall similarity of 88%. The 5' flanking sequences are 77% similar, the coding regions are 91% similar and the 3' untranslated regions up to nucleotide 2,020 of AH119 beyond which the sequence for human *c-jun* is not known are 89% similar (not shown). It is interesting that the 5' flanking sequence of human *c-jun* and mouse *c-jun* has diverged more than the rest of the molecule. Comparison of AH119 amino acid sequence with another member of the JUN family, JUN B (ref. 13 and our unpublished results) shows that they are 52% similar. Only stretches of similarity are seen in the first 230 amino acids, but in the region of the putative DNA binding domain (amino acids 229 to 327 in AH119) the similarity is 75% (not shown). The nucleotide sequences of AH119 and *jun* B are not significantly similar in the 5' flanking and 3' untranslated regions; but in the coding region they are 68% similar (not shown). The amino

acid sequences of AH119, JUN B, and v-JUN contain clusters of highly conserved residues in the amino terminal part of the protein, especially between amino acids 69 and 125 of AH119 (40 identities in 56). Interestingly, between residues 126 and 225 there are only 10 identities. This is followed by the region of the putative DNA binding domain where 72 identities are present in a stretch of 98 amino acid (not shown).

To determine the mRNA levels of AH119 during the G<sub>0</sub>/G<sub>1</sub> transition, RNA was isolated from quiescent NIH 3T3 cells serum-stimulated for different periods of time and analyzed after transfer to Northern blots by hybridization with the complete AH119 insert. The level of AH119 mRNA was undetectable in quiescent cells, but increased dramatically 30 min after stimulation, reaching its maximum level, representing an increase of ~30 fold over that seen in non-stimulated cells after 1 h (Fig. 3a). The mRNA levels decreased slowly up until 4 h after stimulation, remaining at that level for at least 8 h (not shown). Two mRNAs, of estimated sizes 2.7 and 3.2 kb, could be detected with the complete AH119 probe. The overall induction of AH119 mRNA is very similar to that of *c-myc* mRNA and differs significantly from that of *c-fos* mRNA (not shown). The induction of *c-fos* is more rapid and transient, the mRNA is undetectable by 2 h after stimulation. The induction of AH119 mRNA







**Fig. 4** Transcriptional activation of AH119. Nuclei were isolated from quiescent cells (Q) or cells stimulated with serum in the absence (FCS) or presence (FCS+CHX) of cycloheximide at different times and their transcriptional activity was determined by nuclear run-on assays. The labelled transcripts were hybridized against 1 µg of recombinant pUC19 containing the complete AH119 cDNA spotted onto Gene Screen Plus membrane. Nuclei of NIH 3T3 cells were isolated and run-on experiments were done as described<sup>4,20</sup>.

described here is consistent with the recent observation that PEA-1 binding activity (mouse homologue of human AP-1) is stimulated by serum in quiescent cells and that it requires *de novo* protein synthesis<sup>23</sup>. Addition of serum in the presence of cycloheximide led to an induction of both mRNAs 20-fold higher than that observed in the absence of the protein synthesis inhibitor (Fig. 3a). This effect, known as superinduction, is characteristic of genes whose mRNAs have very short half-lives. The ability of different growth factors to increase AH119 mRNA levels was also determined, the most active inducer being platelet-derived growth factor which increased both mRNAs to levels higher than those observed after serum treatment. Bombesin, epidermal growth factor, and insulin were weak inducers (not shown).

To exclude the possibility that the two mRNAs detected by the complete AH119 probe was due to crosshybridization with a related transcript, Northern blots were hybridized with probes generated from the 5' flanking region, coding domain, and 3' untranslated region, corresponding to nucleotides 1-310, 400-800, and 2,130-2,570 respectively. The results demonstrate that probes from the 5' coding region recognize both transcripts, but that the probe from the 3' region only recognizes the larger mRNA (Fig. 3b). This would suggest that both mRNAs are transcripts from the same gene, but that the smaller transcript derives from use of one of the additional polyadenylation sites found in the proximity of nucleotide 2,000 in AH119. The ratio of these transcripts, (the smaller is ~5 times more abundant) is maintained during the induction period, even in the presence of cycloheximide. To obtain further evidence that the two transcripts were products of the same gene, Southern blots were performed with total mouse genomic DNA digested with four different restriction enzymes and hybridized against probes corresponding to the 5' and 3' regions and the complete sequence of AH119 cDNA. In all cases the three probes detected the same fragment, the smallest being 5 kb (not shown). This strongly suggests that the two RNAs detected in Northern analysis are products of the same gene and that the size of the mouse *c-jun* gene may be <5 kb, containing small introns, if any, in the region corresponding to AH119.

The superinduction observed with cycloheximide and the presence of the ATTTA sequence in the 3' untranslated region of AH119 suggested that the mRNA could be an unstable molecule. To investigate this, quiescent cells stimulated with serum in the presence of cycloheximide for 4 h were washed three times with medium and incubated for different periods of time in serum containing actinomycin D in the absence or presence of cycloheximide. The results, illustrated in Fig. 3c, showed that in the absence of protein inhibitor the half-life of AH119 mRNA was 10-15 min and that cycloheximide prolonged it ~fivefold. The behaviour of both transcripts was identical.

To determine whether the changes in AH119 mRNA levels observed after stimulation were due to transcriptional activation

of the gene, nuclear run-on analyses were performed<sup>4,20</sup>. Nuclei from quiescent cells stimulated with serum in the absence or presence of cycloheximide were isolated at different times and the transcriptional activity of AH119 gene was assayed using a dot-blot procedure. A maximal induction of ~100-fold was observed 15 min after stimulation, and activity then decreased rapidly up until 2 h after stimulation and retained a low level for at least 8 h. The presence of cycloheximide prevented the decrease in transcription, maintaining it at very high levels up until 8 h. This, together with the stabilization of AH119 mRNA by cycloheximide could explain the superinduction observed in the presence of protein synthesis inhibition.

The expression of AH119 was examined in different adult mouse organs by Northern blot analysis of poly(A)<sup>+</sup> mRNA hybridized against a complete probe. Two mRNAs of identical size to those detected in stimulated NIH 3T3 cells were initially observed only in mRNA samples of lung, though its level of expression was at least 10-fold lower than in stimulated 3T3 cells (not shown). Longer exposure of the filter showed weak signals (10-fold lower than in lung) in RNAs extracted from brain, thymus, intestine, and testes. In the remaining tissues (liver, heart, kidney, spleen, ovary) the level of AH119 mRNA was undetectable. The significance of the higher level of expression in lung tissue is not clear, but demonstrates that *c-jun* expression is controlled in some mouse tissues.

These findings add another member to the increasing list of putative transcription factors induced in quiescent cells during the G<sub>0</sub> to G<sub>1</sub> transition. It is possible to envisage that these factors, on their own or in complexes, will induce or repress genes essential for progression of the cell-cycle through G<sub>1</sub>. Some of the induced gene products could be essential for the G<sub>0</sub>/G<sub>1</sub> transition as described for c-FOS<sup>24</sup> or for normal cell proliferation as demonstrated for c-MYC<sup>25</sup>, or both.

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# Epidermal growth factor stimulates transcription of the *c-jun* proto-oncogene in rat fibroblasts

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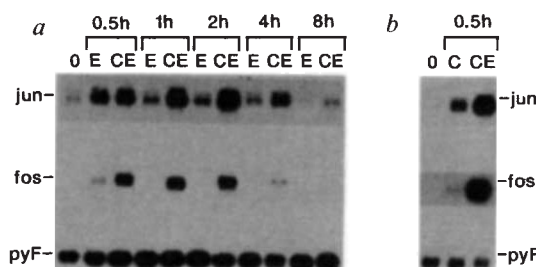
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Some growth factor-induced genes, such as the *c-fos* gene, are activated rapidly and transiently without intervening protein synthesis<sup>1</sup>. Others, like the rat transin gene<sup>2</sup>, are activated more slowly but more durably and their activation requires prior protein synthesis. It is tempting to speculate that certain rapidly-activated genes code for transcription factors which interact directly with promoter regions of genes like the transin gene to trigger their expression. Unfortunately, little is known about the majority of primary response genes to support this hypothesis. The proto-oncogene *c-jun* codes for the transcription factor AP-1 or a closely related protein<sup>3,4</sup>. We show that epidermal growth factor stimulates transcription of the *c-jun* gene in fibroblasts as a primary response. This supports the notion that increased expression of genes encoding transcription factors is an important element of the signal transduction mechanism, assuring the long-term transcriptional response of cells to growth factors.

A *c-jun* complementary DNA clone was obtained by screening a human tumour cDNA library<sup>5</sup> with a *v-jun*<sup>6</sup> specific probe. The sequence of this 1.1 kilobase cDNA showed that it contained the entire protein-coding sequence (the cDNA contains nucleotides 352–1442 of the published sequence<sup>4</sup>). The *c-jun* cDNA detects a single fragment (data not shown) in *Eco*RI or *Hind*III digests of rat genomic DNA under high-stringency conditions ( $0.1 \times \text{SSC}$ ,  $0.1\%$  SDS at  $50^\circ\text{C}$ ;  $1 \times \text{SSC} = 0.15 \text{ M NaCl}$ ,  $0.015 \text{ M Na citrate pH } 7$ ), and so is suitable for use in detecting rat *c-jun* messenger RNAs. Indeed, under the same conditions the probe detects rat RNAs of 2.5 and 3.2 kb (Fig. 1 and data not shown). This behaviour is reminiscent of that seen with human RNA, where *c-jun* species of 2.6–2.7 kb have been described<sup>3,4</sup> with a minor 3.4 kb component believed to be a precursor.

Treatment of quiescent, serum-starved Rat-1 fibroblasts with epidermal growth factor (EGF) results in a rapid, transient increase in *c-jun* mRNA levels (Fig. 1a). An 8-fold increase is observed after 30 min of stimulation; levels 60 min after stimulation are about twofold above those in uninduced cells. In the presence of the protein synthesis inhibitor cycloheximide a more durable superinduction (greater than 20-fold) by EGF is seen (Fig. 1a). Cycloheximide when added alone also increases *c-jun* mRNA levels somewhat (~fivefold, Fig. 1b). It is now well established that growth factor treatment of cells generally leads to a rapid, transient increase in expression of the *c-fos* proto-oncogene, and that this increase is enhanced in the presence of cycloheximide<sup>1,7–11</sup>. As shown in Fig. 1, this behaviour is also observed in Rat-1 cells. Comparison of the results obtained for *c-jun* and *c-fos* mRNAs shows that they are broadly similar, although *c-jun* mRNA basal levels are higher and the EGF-response is somewhat longer lived.

To test if the EGF-induced increases in *c-jun* mRNA levels reflect an enhanced rate of transcription of the corresponding gene, we performed run-off transcription experiments using isolated nuclei from Rat-1 cells. As shown in Fig. 2, EGF stimulation of quiescent Rat-1 cells leads to increased transcription of the *c-jun* gene (sevenfold and fivefold increases after 15 min and 30 min respectively). EGF-stimulation in the presence of cycloheximide leads to a further elevation in transcription levels (12- and 16-fold increases after 15 and 30 min respectively). Treatment with cycloheximide alone only slightly



**Fig. 1** Accumulation of *c-jun* and *c-fos* mRNAs. *a*, Kinetics of mRNA accumulation following treatment with EGF in the presence or absence of cycloheximide. *b*, Effect of cycloheximide alone. 0, uninduced cells; E, cells induced with EGF; C, cells treated with cycloheximide; CE, cells induced with EGF in the presence of cycloheximide.

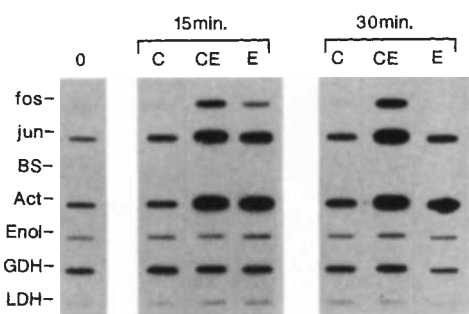
**Methods.** Confluent monolayers of Rat-1 cells were washed twice with phosphate-buffered saline (PBS) and incubated for 14 h in serum-free medium as described elsewhere<sup>18</sup>, before treatment with EGF ( $20 \text{ ng ml}^{-1}$ , BRL), cycloheximide ( $10 \mu\text{g ml}^{-1}$ ) or EGF in the presence of cycloheximide. Total RNA samples were prepared by the LiCl-urea technique<sup>19</sup>, electrophoresed ( $10 \mu\text{g}$  aliquots) on a 1.2% formaldehyde-agarose gel<sup>20</sup> and transferred to Hybond-N membranes (Amersham). Prehybridizations, hybridizations and washings were carried out as described<sup>5</sup>. Nick-translated probes used were a cloned human *c-jun* cDNA (jun, see text), and a 1.6 kb *Bgl*III fragment carrying *v-fos* sequences<sup>21</sup> (fos). Filters were subsequently rehybridized to a cloned cDNA picked at random from a polyoma virus-transformed rat embryo fibroblast cell line cDNA library<sup>18</sup> (pyF) to correct for possible variations in amounts of RNA loaded on the gel or transfer efficiency. Filters were exposed to X-ray film and autoradiograms obtained were scanned. The ratio of the jun signal to the pyF signal was calculated for each sample and used to determine the magnitude of inductions.

increases transcription (a twofold increase after 15 or 30 min). Use of single-stranded recombinant M13-*c-jun* probes to assay for transcription of either strand separately indicates that only the sense strand of the *c-jun* gene is transcribed (data not shown). Transcription of the gene is blocked by  $1 \mu\text{g ml}^{-1}$   $\alpha$ -amanitin, as expected for a gene transcribed by RNA polymerase II (data not shown). As for the increases in *c-jun* mRNA levels (Fig. 1), the increased transcription of the *c-jun* gene is accompanied as expected<sup>7</sup> by increased transcription of the *c-fos* gene, and also of an actin gene. However, transcription of genes coding for three glycolytic enzymes remains essentially unchanged during these experiments (Fig. 2, Enol, GDH, LDH).

We conclude that the rapid but transient increase in *c-jun* mRNA levels induced by EGF in Rat-1 cells is due at least in part to increased transcription of the gene. This increase appears not to require new protein synthesis. Indeed, maximal levels of transcription are obtained with EGF in the presence of cycloheximide, perhaps due to stabilization of a transcriptional activator or inhibition of production of a labile repressor.

The *c-jun* gene appears to code for the transcription factor AP-1 (refs 3, 4). AP-1 interacts with specific enhancer elements which can confer responsiveness to phorbol ester tumour promoters<sup>12,13</sup> on heterologous promoters. The growth factor-induced increases in the levels of AP-1 implied by our data may be important to trigger expression of a class of genes with AP-1 binding sites following growth factor stimulation. For example, EGF-induction of the rat transin gene, which contains an AP-1-binding site<sup>12,13</sup> in its promoter region<sup>2</sup>, requires preliminary synthesis of new proteins. One of these proteins might be AP-1. Activation of other growth factor-induced genes could require prior synthesis of distinct transcription factors. For example, the *jun-B* gene<sup>14</sup>, which is also regulated directly by growth factors, codes for a JUN-related protein of unknown function but which could be a transcription factor with a different binding specificity to that of AP-1. Finally, it is interesting to note the similarities between the responses of the *c-jun* and *c-fos* genes to EGF. The *c-fos* protein complex binds, directly or indirectly,





**Fig. 2** Transcription analysis of genes after stimulation of Rat-1 cells. Plasmids bound to nitrocellulose were hybridized with  $^{32}\text{P}$ -labelled run-off transcripts from nuclei isolated at different times after treatment with cycloheximide (C), EGF (E) or EGF plus cycloheximide (EC). 0 = untreated cells. In preparing the nitrocellulose filters, the following plasmids or fragments were used: fos: a 1.6 kb *Bgl*II fragment containing v-fos sequences<sup>21</sup>. jun: a 1.1 kb c-jun cDNA fragment cloned in the plasmid Bluescript M13- (Stratagene). BS: Bluescript M13- plasmid. Actin: pACT-2, containing rat actin cDNA<sup>22</sup>. Enol: pENO-2, containing rat enolase cDNA<sup>22</sup>. GDH: pGAPDH-2, containing rat glyceraldehyde-3-phosphate dehydrogenase cDNA<sup>22</sup>. LDH: pLDH-2, containing a rat lactate dehydrogenase cDNA<sup>22</sup>.

**Methods.** Cells were prepared and treated as in the legend to Fig. 1. Nuclei isolation and run-off transcriptions were carried out as described previously<sup>18</sup>. DNA (2  $\mu\text{g}$ ) was heat-denatured before application to nitrocellulose using a slot blot apparatus. Hybridizations were carried out as in ref. 7 with minor modifications (R. Nicholson, personal communication). Filters were washed extensively in  $0.1 \times \text{SSC}$  containing 0.1% SDS at  $50^\circ\text{C}$  before treatment with pancreatic ribonuclease A ( $10 \mu\text{g ml}^{-1}$ ) in  $2 \times \text{SSC}$  at  $25^\circ\text{C}$  and rinsing with  $2 \times \text{SSC}$  at room temperature. Filters were exposed to X-ray film and autoradiograms obtained were scanned. The ratio of the jun signal to the enolase signal was calculated for each sample and used to determine the magnitude of changes in transcription.

to AP-1-binding sites<sup>15-17</sup>. It is conceivable that this binding involves AP-1, which could provide a rationale for growth factor increased expression of both *c-fos* and *c-jun* genes. Evidence supporting this hypothesis comes from the recent finding that the product of the *c-jun* gene is the FOS associated protein p39 (ref. 23). Intriguingly, the promoter of the *c-fos* gene contains a sequence closely resembling an AP-1 binding site (ref. 24), suggesting that in addition to forming a complex with FOS the JUN protein might under certain circumstances be involved in controlling expression of the *c-fos* gene.

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## Thyroid hormone receptor $\alpha$ isoforms generated by alternative splicing differentially activate myosin HC gene transcription

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Thyroid hormones are thought to modulate gene expression positively or negatively through interactions with chromatin-associated receptors<sup>1</sup>. Recently, the *c-erbA* proto-oncogene products have been shown to be nuclear thyroid hormone ( $\text{T}_3$ ) receptors ( $\text{TR}$ )<sup>2-5</sup> by sequence similarity with other steroid receptors and by their ability to bind thyroid hormone. But it has not been shown that these receptors directly activate transcription of the responsive genes *in vivo*. In addition, the rat  $\text{TR}\alpha$  gene encodes several messenger RNA (mRNA) species, generated by differential processing of its transcripts (ref. 22). For these reasons we investigated the ability of two major isoforms of the rat  $\text{TR}\alpha$  gene products to activate transcription of a sarcomeric myosin heavy chain (mHC) gene, because expression of all members of this gene family is responsive to  $\text{T}_3$ <sup>6</sup>. We show here that the  $\text{rTR}\alpha 1$  receptor is a thyroid hormone-dependent transcriptional factor, which upon binding the  $\text{T}_3$  responsive element of the  $\alpha$ -mHC gene, activates expression of this gene *in vivo*. The  $\text{rTR}\alpha 2$  isoform, which is identical to  $\text{rTR}\alpha 1$  except for its carboxyl terminal portion, is generated by alternative splicing of the  $\text{rTR}\alpha$  gene transcript. This peptide, when produced *in vitro* and *in vivo* failed to bind  $\text{T}_3$  or other hormones or to *trans-activate*  $\alpha$ -mHC gene expression. Thus, alternative splicing can produce marked differences in the functional properties of a transcriptional factor.

Characterization of five rat cardiac complementary DNA clones, isolated using the avian *v-erbA* gene as a probe, demonstrated that these clones correspond to three distinct mRNAs produced by alternative splicing of a common  $\text{TR}\alpha$  gene transcript<sup>22</sup>. One of the cDNA clones is essentially identical to that previously isolated from a rat brain library<sup>4</sup> and corresponds to a protein of 410 amino acids,  $\text{rTR}\alpha 1$ . The two other cDNA clones diverge from each other only in their respective 5' untranslated end sequences, and specify the same 492 amino-acid-long polypeptide,  $\text{rTR}\alpha 2$ . The sequence of  $\text{rTR}\alpha 2$  is identical to that of  $\text{rTR}\alpha 1$  up to amino acid 370 but quite different in its carboxyl terminal portion, which is 82-amino acids longer than that of  $\text{rTR}\alpha 1$  (Fig. 1). When compared with known thyroid/steroid hormone receptors and other related gene products (see refs 7, 8), this sequence is most similar to the human testis thyroid hormone receptor *hc-erbA-T* (refs 5, 9), but has 12 amino acid differences clustered at the carboxyl end (Fig. 1).

The structural differences seen in the carboxyl-terminal coding portion of  $\text{rTR}\alpha 1$  and  $\text{rTR}\alpha 2$ , which includes the putative ligand-binding 'E' domain of the proteins, led us to analyse the functional properties of these two isoforms. We synthesised  $\text{rTR}\alpha 1$  and  $\text{rTR}\alpha 2$  mRNA transcripts with SP6 polymerase and translated them *in vitro*. Both mRNAs were translated with similar efficiency and produced peptides of relative molecular mass ( $M_r$ )  $\sim 53,000$  (53K) and 64K respectively, consistent with

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|     |                                                        |                  |
|-----|--------------------------------------------------------|------------------|
| 1   | MEQPKSKVEC GSDPEENSAR SPDGKRKRKN GQCPLKSSMS GYIPSYLDKD | rTR $\alpha$ 1/2 |
| 51  | EQCVVCQDKA TGYHYRCITC EGCKGFFRRT IQKNLHPTYS CKYDSCCVID | rTR $\alpha$ 1/2 |
| 101 | KITRNQCQLC RFKKCIAVGM AMDLVDDSK RVAKRKLIEQ NRERRRKEEM  | rTR $\alpha$ 1/2 |
| 151 | IRSLQQRPEP TPEEWDLIHW ATEAHRSTNA QGSHWKRRK FLPDDIGQSP  | rTR $\alpha$ 1/2 |
| 201 | IVSMPDGDV DLEAFSEFTK IITPAITRVV DFAKKLPMS ELPCEQIIL    | rTR $\alpha$ 1/2 |
| 251 | LKGCCMEIMS LRAAVRYDPE SDTLTSGEM AVKRKQLKNG GLGVVSDAIF  | rTR $\alpha$ 1/2 |
| 301 | ELGKSLSAFN LDDTEVALLQ AVLLMSTDRS GLLCVDKIEK SQEAYLLAFE | rTR $\alpha$ 1/2 |
| 351 | HYVNRKHNI PHFWPKLLMK                                   | rTR $\alpha$ 1   |
|     | VTDLRMIGAC HASRFLHMKV ECPTELFPL                        | rTR $\alpha$ 1   |
|     | EREVQSSILY KGAAAEGRPG GSLGVHPEGQ                       | rTR $\alpha$ 2   |
| 401 | QLLGMHVVGQ                                             | rTR $\alpha$ 1   |
|     | FLEVFEDQEV PQVRQLEQF GEAGSLRGPV LQHSPKSPQ QRLELLHRS    | rTR $\alpha$ 2   |
| 451 | GILHSRAVCG EDDSSSEASSL SSSSSDEDE VFEDLAGKAA SP         | rTR $\alpha$ 2   |
|     | A D P **E EP C N                                       |                  |

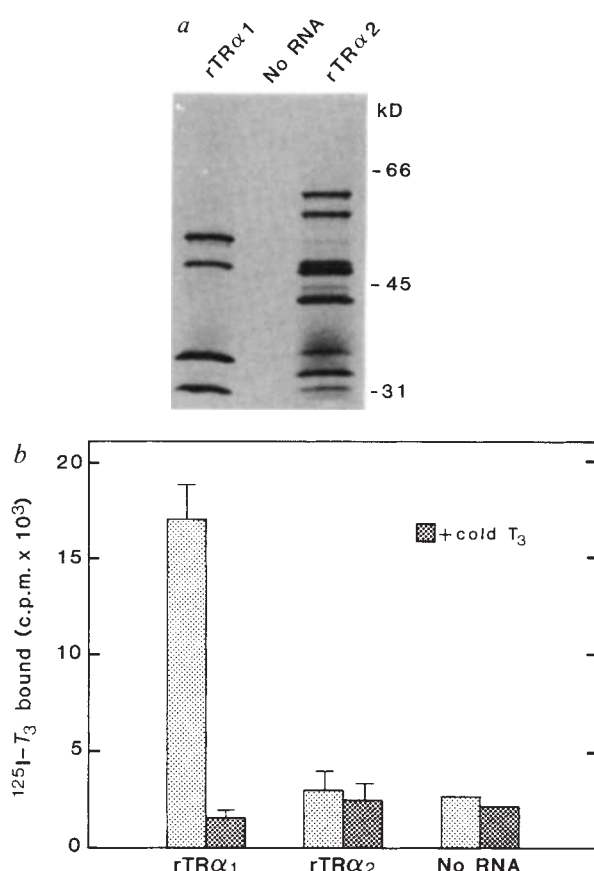
**Fig. 1** Predicted amino acid sequence of rTR $\alpha$ 1 and rTR $\alpha$ 2. Common and isoform-specific carboxyl terminal amino acid sequences are displayed. rTR $\alpha$ 1 sequence is essentially identical to the rat brain rTR $\alpha$  sequence<sup>4</sup>, except for one amino acid change at position 281 (A, instead of T)<sup>4</sup>. The rTR $\alpha$ 2 sequence is most similar to hc-erbA-T<sup>5</sup>. Residues that differ from those in hc-erbA-T are indicated underneath their corresponding positions. Asterisks indicate absence of the corresponding residues in the human sequence.

**Methods.** Polyadenylated RNA was isolated from pooled hearts of two-month-old rats and the oligo dT primed cDNA library was constructed in  $\lambda$ gt10 as described elsewhere<sup>11</sup>. The library was screened with the 500-base-pair (bp) long *Pst*I fragment of *v-erbA*, labelled with [ $\alpha$ -<sup>32</sup>P]-dCTP by random oligonucleotide priming<sup>12</sup>. The cDNA inserts were subcloned into the *Eco*RI site of pGEM3 vector (Promega Biotec) and the nucleotide sequence determined by the dideoxy chain termination method<sup>13</sup>.

410 and 492 amino-acid-long proteins (Fig. 2a). These translation products were used in T<sub>3</sub> binding assays (Fig. 2b). The rTR $\alpha$ 1 peptides bound specifically to <sup>125</sup>I-labelled T<sub>3</sub>, as demonstrated by competition in excess of unlabelled hormone (and see ref. 4) but the rTR $\alpha$ 2 peptides did not. Other thyroid hormone analogues, such as T<sub>4</sub>, reverse T<sub>3</sub>, and other hormones, such as testosterone, vitamin D<sub>3</sub> and retinoic acid, all failed to bind the rTR $\alpha$ 2 peptides (not shown).

To test the function of rTR $\alpha$ 1 and rTR $\alpha$ 2 *in vivo*, the corresponding cDNA sequences were placed under the transcriptional control of the SV40 promoter and transfected into COS cells. Equal levels of pSVrTR $\alpha$ 1 and pSVrTR $\alpha$ 2 transcripts were readily detectable by S1 nuclease mapping analysis, using a 3' end specific rTR $\alpha$ 1 cDNA probe for hybridization (Fig. 3a). Nuclear extracts were prepared to determine the thyroid hormone-binding capacity of the control and transfected COS cells (Fig. 3b). Transient expression of the pSVrTR $\alpha$ 1 produced a five-fold increase in specific <sup>125</sup>I-labelled T<sub>3</sub> binding, compared to that of mock-transfected cells. This value, when corrected for an estimated 5–10% transfection efficiency, indicates a 50–100 fold increase in the level of receptors in the transfected cells. In contrast, no significant change was produced by the transient expression of pSVrTR $\alpha$ 2, corroborating the results of *in vitro* binding assays presented above. Thus, of the protein isoforms encoded by the rTR $\alpha$  gene, one is a thyroid hormone binding protein, the other is not. These results demonstrate that at most twelve amino acid differences, between rTR $\alpha$ 2 and hc-erbA-T<sup>5</sup> are sufficient to abolish the T<sub>3</sub> binding function of the protein, and establish that the functional ligand binding domain of the T<sub>3</sub> receptors resides in their carboxyl termini.

The ability of rTR $\alpha$ 1 to activate gene transcription *in vivo* was assessed using as target the promoter of the rat cardiac  $\alpha$ -myosin heavy chain (mHC) gene which is up regulated by T<sub>3</sub> (ref. 6). The pSVrTR $\alpha$  plasmids were co-transfected into



**Fig. 2** *In vitro* synthesis and thyroid hormone binding assay of rTR $\alpha$ 1 and rTR $\alpha$ 2 proteins. *a*, SDS-polyacrylamide gel analysis of the *in vitro* translated products. Protein size markers used were bovine serum albumin (66K), ovalbumin (45K) and carbonic anhydrase (31K). *b*, Thyroid hormone binding of the *in vitro* translated products. Reticulocyte lysates programmed with the *in vitro* transcripts were incubated with <sup>125</sup>I-labelled T<sub>3</sub> in the presence or absence of 1,000-fold excess of unlabeled L-T<sub>3</sub>. The results are mean  $\pm$  s.d. of three experiments.

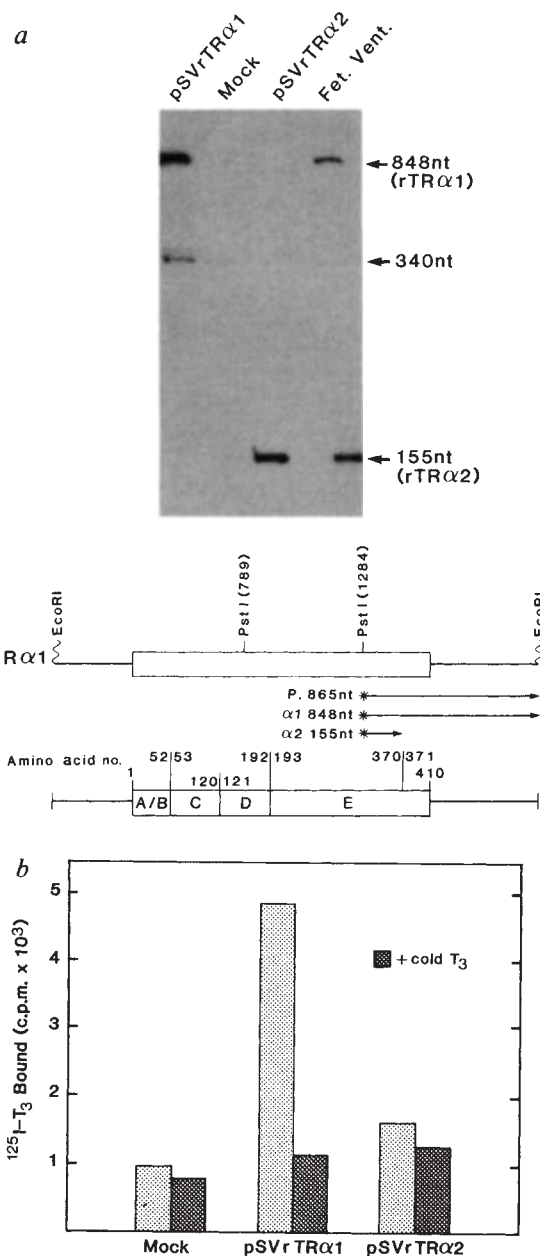
**Methods.** For *in vitro* transcription, the 5' untranslated sequences of the rTR $\alpha$  cDNA inserts were trimmed at their common *Bgl*I site and remaining portions were subcloned into pGEM3 vectors. The *in vitro* transcription was performed using SP6 promoter RNA polymerase in conditions suggested by the manufacturer. The transcription mixture was treated with DNase and the transcripts were purified by Sephadex G50 chromatography. Two  $\mu$ g of *in vitro* transcripts were added to 35  $\mu$ l of rabbit reticulocyte lysate (Promega Biotec) in the presence of <sup>35</sup>S-methionine; 2  $\mu$ l of translation mixture was loaded onto a 7.5% reducing SDS-polyacrylamide gel and the products visualized by autoradiography. For hormone binding assays, 2  $\mu$ l of programmed lysates were mixed with L-<sup>125</sup>I-labelled T<sub>3</sub> (New England Nuclear, 2,200 Ci mM<sup>-1</sup>) in the buffer containing 0.25 M sucrose, 0.25 M KCl, 20 mM Tris-HCl (pH 7.5), 1 mM MgCl<sub>2</sub>, 2 mM EDTA and 5 mM DTT<sup>3</sup>. The mixture was incubated for 16 h at 0 °C and bound radiolabelled hormone was determined by filter binding assay as described<sup>14</sup>. Specific hormone binding was determined by adding 1,000-fold excess of cold L-T<sub>3</sub>.

myogenic Sol 8 cells<sup>23</sup> with a chloramphenicol acetyl transferase (CAT) reporter gene construct, under the control of the  $\alpha$ -mHC gene promoter. By this assay, the putative function of rTR $\alpha$ 2 as a transcriptional factor, in spite of its apparent inability to bind hormones, could be tested (Fig. 4a). In the absence of thyroid hormones, pSVrTR $\alpha$ 1 induced the basal level of expression of the  $\alpha$ -mHC CAT gene construct by less than 40%. A concentration-dependent increase in CAT activity was produced upon addition of T<sub>3</sub> or thyroid hormone analogues. Maximal stimulation (300–600%) was observed at 10<sup>-8</sup> M T<sub>3</sub>, 10<sup>-7</sup> M T<sub>4</sub> and 10<sup>-9</sup> M TRIAC, reflecting the affinity of the TR $\alpha$ 1



**Fig. 3** Transient expression of rTR $\alpha$ 1 and rTR $\alpha$ 2 cDNAs in COS cells. *a*, S<sub>1</sub> nuclease mapping of the RNAs from transfected COS cells. A diagram of the probe is shown, together with a partial restriction map of cDNA clones rTR $\alpha$ 1. *Pst*I restriction endonuclease cleavage sites are indicated with their corresponding coordinates. Lines and open boxes indicate untranslated and coding sequences, respectively. Bottom, schematic diagram of the receptor protein with putative functional domains (A/B, C, D and E, ref. 4). The probe used was the 865-nt long *Pst*I–*Eco*RI fragment of rTR $\alpha$ 1 cDNA. This single-stranded probe encompasses the carboxyl terminal portion and entire untranslated region of the clone, and extends into the polylinker region of the cloning vector. As seen in rat fetal cardiac ventricle (fet. vent) the probe produces a 848 nucleotide (nt) band when hybridized to homologous RNAs. The 155-nt partially-protected band specifies mRNAs, such as rTR $\alpha$ 2, that diverge from the probe at codon 371. A faint 340 nt band detected in all COS cell samples probably represents endogenous TR mRNAs with partial homology to the probe. Each lane received 30  $\mu$ g RNA. Transfections were with 20  $\mu$ g pSVrTR $\alpha$  plasmids as indicated or without DNA, mock transfected cells. S<sub>1</sub> nuclease mapping analysis was performed as described<sup>6</sup>. *b*, Thyroid hormone binding assay on nuclear extracts of transfected COS cells. Cell nuclei were obtained by the method of Dignam *et al.*<sup>15</sup> and extracted in a buffer containing 0.6 M KCl as previously described<sup>10</sup>. Hormone binding assays were performed as described in Fig. 2 legend.

**Methods.** The rTR $\alpha$ cDNA inserts used for *in vitro* transcription (see Fig. 2 legend) were cloned, after addition of *Hind*III linkers at their 5' end, as *Hind*III–*Bam*HI fragments into the restricted pSV2 vector<sup>16</sup> to generate pSVrTR $\alpha$ 1 and pSVrTR $\alpha$ 2 under the transcriptional control of the SV40 promoter. Transfection conditions were as reported previously<sup>17</sup>.



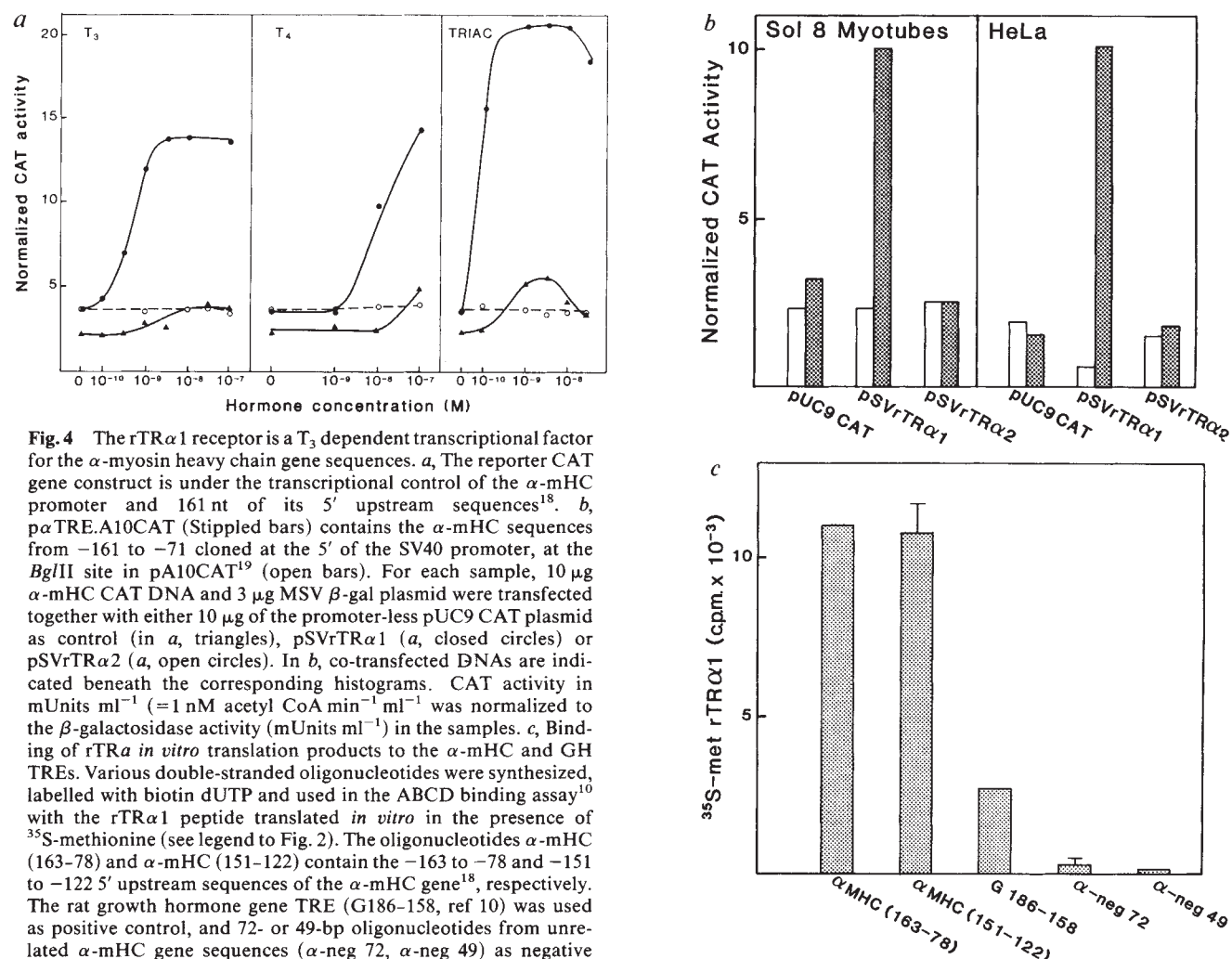
receptor for the respective hormones<sup>4</sup>. These data establish unambiguously rTR $\alpha$ 1 as a thyroid rTR $\alpha$ 1 as a transcriptional factor that is dependent on thyroid hormones for activating gene expression. In contrast, although cotransfection of pSVrTR $\alpha$ 2 also induced  $\alpha$ -mHC CAT expression in the absence of thyroid hormones by 40%, no further induction was produced by thyroid hormones, in any condition tested. To our knowledge, this is the first demonstration that the function of a transcriptional factor can be dramatically altered by swapping its ligand binding domain by alternative pre-mRNA splicing.

To characterize further the interactions between rTR $\alpha$ 1 and  $\alpha$ -mHC gene sequences, we mapped the thyroid-responsive element (TRE) in this gene. By deletion analysis, we found that T<sub>3</sub> inducible expression of the  $\alpha$ -mHC was conferred by 5' upstream sequences between positions -161 and -71 (ref. 23). When these sequences were introduced to the 'enhancerless' pA10CAT plasmid, the transcriptional activity of the SV40 promoter in the resulting p $\alpha$ TRE.A10CAT construct became T<sub>3</sub> inducible, in muscle as well as non-muscle cells, when co-expressed with the pSVrTR $\alpha$ 1 plasmid (Fig. 4b). The level of inducibility of p $\alpha$ TRE.A10CAT was comparable to that of the

$\alpha$ -mHC gene construct, demonstrating that the  $\alpha$ -mHC TRE is necessary and sufficient to confer T<sub>3</sub> responsiveness and is neither promoter- nor cell-specific.

To determine whether *trans*-activation of the  $\alpha$ -mHC gene involves direct receptor-DNA interactions, synthetic oligonucleotides corresponding to the  $\alpha$ -mHC TRE were used in the avidin-biotin complex DNA (ABCD) binding assay<sup>10</sup>, using the *in vitro* translated rTR $\alpha$  plasmids. As shown in Fig. 4c, rTR $\alpha$ 1 bound specifically to the  $\alpha$ -mHC TRE sequences, with an apparent affinity even higher than for the rat growth hormone TRE sequences<sup>10</sup>.

The functional analyses presented here clearly demonstrate that rTR $\alpha$ 1 is a thyroid hormone-dependent transcriptional factor that activates expression of the  $\alpha$ -mHC gene. They also establish that positive regulation of the two T<sub>3</sub> responsive genes so far analysed (This report and ref. 10) occurs through a common mechanism, involving a direct interaction of the T<sub>3</sub> receptors with a consensus *cis*-regulatory gene sequence. Indeed, nucleotide sequence comparison of  $\alpha$ -mHC TRE with the TR binding domain in the growth hormone gene<sup>10</sup>, pointed to a conserved 13 nucleotide (nt) core sequence element ( $\alpha$ -mHC:



**Fig. 4** The rTR $\alpha$ 1 receptor is a T<sub>3</sub> dependent transcriptional factor for the  $\alpha$ -myosin heavy chain gene sequences. **a**, The reporter CAT gene construct is under the transcriptional control of the  $\alpha$ -mHC promoter and 161 nt of its 5' upstream sequences<sup>18</sup>. **b**, p $\alpha$ TRE.A10CAT (Stippled bars) contains the  $\alpha$ -mHC sequences from -161 to -71 cloned at the 5' of the SV40 promoter, at the Bgl/II site in pA10CAT<sup>19</sup> (open bars). For each sample, 10  $\mu$ g  $\alpha$ -mHC CAT DNA and 3  $\mu$ g MSV  $\beta$ -gal plasmid were transfected together with either 10  $\mu$ g of the promoter-less pUC9 CAT plasmid as control (in **a**, triangles), pSVrTR $\alpha$ 1 (**a**, closed circles) or pSVrTR $\alpha$ 2 (**a**, open circles). In **b**, co-transfected DNAs are indicated beneath the corresponding histograms. CAT activity in mUnits ml<sup>-1</sup> (=1 nM acetyl CoA min<sup>-1</sup> ml<sup>-1</sup>) was normalized to the  $\beta$ -galactosidase activity (mUnits ml<sup>-1</sup>) in the samples. **c**, Binding of rTR $\alpha$  in *in vitro* translation products to the  $\alpha$ -mHC and GH TREs. Various double-stranded oligonucleotides were synthesized, labelled with biotin dUTP and used in the ABCD binding assay<sup>10</sup> with the rTR $\alpha$ 1 peptide translated *in vitro* in the presence of <sup>35</sup>S-methionine (see legend to Fig. 2). The oligonucleotides  $\alpha$ -mHC (163-78) and  $\alpha$ -mHC (151-122) contain the -163 to -78 and -151 to -122 5' upstream sequences of the  $\alpha$ -mHC gene<sup>18</sup>, respectively. The rat growth hormone gene TRE (G186-158, ref 10) was used as positive control, and 72- or 49-bp oligonucleotides from unrelated  $\alpha$ -mHC gene sequences ( $\alpha$ -neg 72,  $\alpha$ -neg 49) as negative controls.

**Methods.** Cells were maintained in culture as described<sup>17</sup> in hormone-depleted serum<sup>20</sup>. Thyroid hormones added after glycerol shock were at the indicated concentrations (**a**), or 30 nM T<sub>3</sub> (**b**). CAT activity in the cell extracts was determined using a recently established protocol<sup>21</sup>. For ABCD binding assay, oligonucleotides with complementary 3' ends were synthesized (Biosearch Scientific) and annealed. The overhanging 5' ends were filled with a deoxynucleotide mixture containing biotin 11-dUTP in place of dTTP, using the large fragment of *Escherichia coli* DNA polymerase. The oligonucleotides were designed so that a total of seven biotin residues were incorporated at both ends or each double-stranded DNA. The concentration of double-stranded oligonucleotides was determined by fluorometer. DNA-receptor binding was performed using 100 fmol of DNA and 4  $\mu$ l of reticulocyte lysate programmed with *in vitro* transcripts of rTR $\alpha$ 1 cDNA, in which the 5' untranslated sequence had been deleted to augment translation efficiency. The binding reaction was in 10 nM L-T<sub>3</sub>, 200  $\mu$ g ml<sup>-1</sup> poly dI.dC, 50 mM KCl, 20 mM HEPES (pH 7.8), 1 mM ZnCl<sub>2</sub>, 1 mM  $\beta$ -mercaptoethanol, 1 mM EDTA, 0.1% Nonidet P-40 and 20% glycerol, at 22 °C for 60 min. Protein-DNA complexes were precipitated by addition of streptavidin-agarose (BRL)<sup>10</sup>.

5'-GGAGGTGACAGGA3' located at positions -132 to -144 of the anti-sense strand, GH: 5'-GGACGTGACCGCA-3' at -176 to -164 of the sense strand). In addition, the rTR $\alpha$  gene can generate, by alternative splicing, another isoform which does not appear to have any of the functional properties of the rTR $\alpha$ 1 receptor. A puzzling observation is the abundance of the rTR $\alpha$ 2 mRNA in all tissue examined, especially in brain and testis (ref. 22), where the thermogenic effect elicited by thyroid hormones is noticeably lacking<sup>1</sup>. This suggests that this isoform may have an as yet undetermined physiological role.

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# The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain

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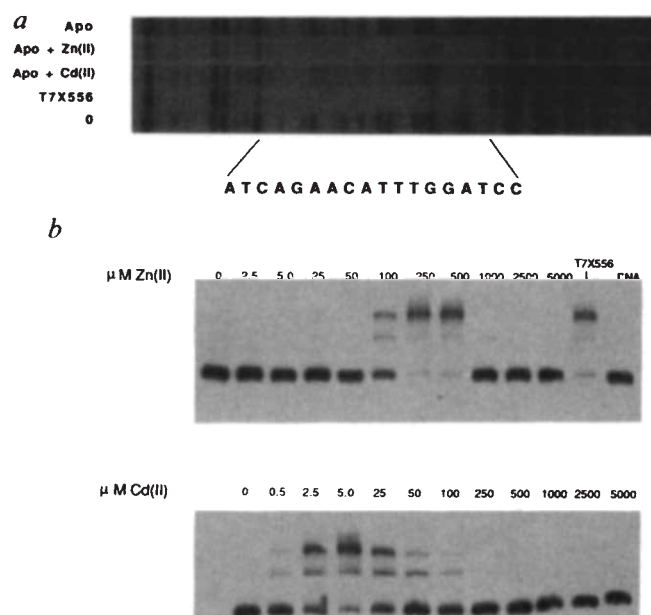
The glucocorticoid receptor enhances or represses transcription by binding to specific DNA sequences termed glucocorticoid response elements, or GREs<sup>1-5</sup>. Studies of cloned glucocorticoid receptors<sup>6-8</sup> reveal that the protein is organized as functional domains, in an arrangement that appears to be common among members of the steroid receptor family<sup>9</sup>. A segment near the centre of the gene specifies DNA binding activity *in vitro* and contains two sequence motifs similar to 'zinc fingers' found in *Xenopus* transcription factor IIIA (TFIIIA)<sup>10</sup>. Such sequence motifs have been identified in nucleic acid binding proteins from a wide range of organisms<sup>11-13</sup>. Steroid receptor protein fingers are proposed to bind zinc through two pairs of conserved cysteine residues<sup>7,14,15</sup>.

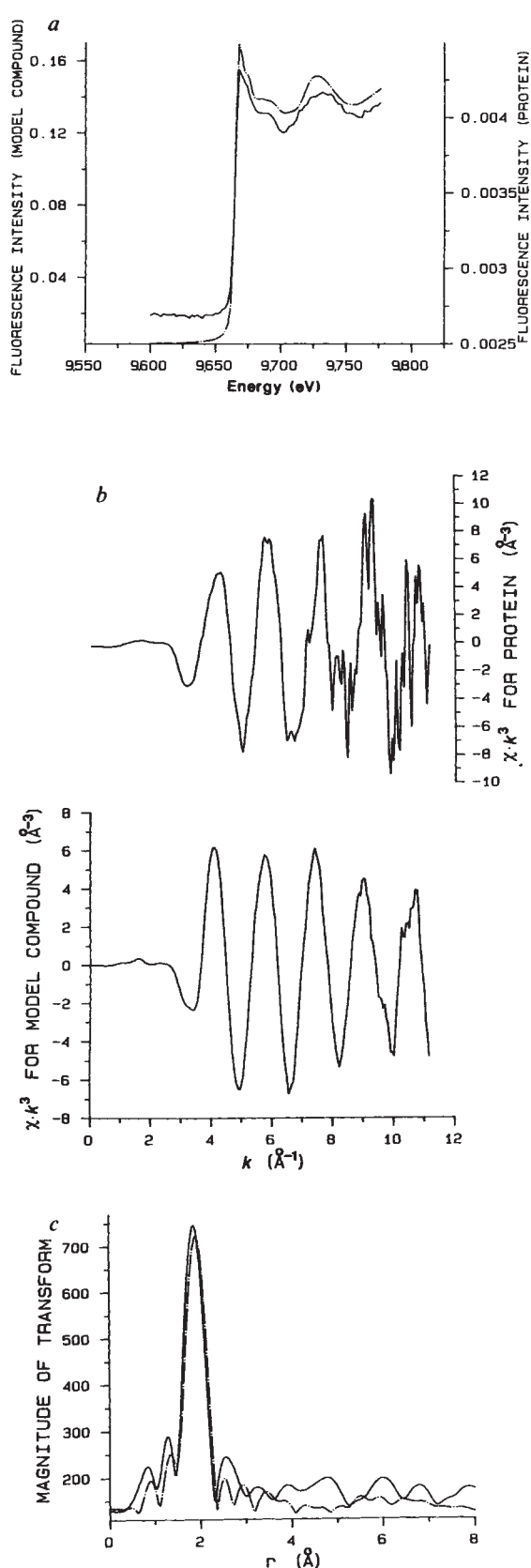
**Fig. 1** Specific binding of T7X556 to GRE sequences requires zinc or cadmium. **a**, ApoT7X556 requires metal for sequence-specific DNA binding. Apo-protein (600 ng) was mixed with water, ZnCl<sub>2</sub> to 250  $\mu$ M, or CdSO<sub>4</sub> to 5  $\mu$ M and incubated on ice for 30–60 min. DNase I footprinting was carried out essentially as described<sup>22</sup> using a <sup>32</sup>P-labelled 250-base-pair fragment containing a 27-bp GRE. Control lanes show footprint patterns obtained with no added protein and with untreated T7X556. **b**, GRE DNA binding activity by T7X556 as a function of Zn(II) and Cd(II) concentration. Apo-T7X556 (100 ng) was reconstituted with the indicated concentrations of ZnCl<sub>2</sub> or CdSO<sub>4</sub> and assayed for specific DNA binding by gel mobility shift<sup>23</sup> using the same labelled probe as in **a**. Untreated T7X556 was also assayed for DNA binding; 'DNA' denotes input DNA alone.

**Methods.** Strategy for overexpression of T7X556 in *E. coli*. Receptor sequences encoding amino acids 407–556, plus a polylinker at the C-terminus encoding an additional 14 nonreceptor residues, were cloned into the unique *Bam*HI site of the T7 RNA polymerase-dependent expression vector pAR3040 (ref. 16) forming an in-frame fusion with the first ten amino acids of T7 gene 10. This plasmid was used to transform BL21(DE3)/pLysS, a strain carrying a stable integrant of T7 gene 1 (RNA polymerase) under the control of the *lac* UV5 promoter<sup>16</sup>. T7 RNA polymerase was induced at mid-logarithmic growth by addition of IPTG to 0.5 mM, resulting in expression of T7X556. Three hours after induction, cells were collected by centrifugation. After freezing and thawing, cell pellets were resuspended in three volumes of lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 10% glycerol, 5 mM DTT, 500 mM NaCl). After 15 min on ice, sodium deoxycholate was added to 0.05%, and the sample was stirred at 4 °C for 30 min, followed by centrifugation at 60,000g for 60 min at 4 °C. Nucleic acids were removed from the supernatant by adding poly(ethylenimine) to 0.2% over a 10 min period, followed by centrifugation at 35,000g at 4 °C for 30 min. Ammonium sulphate was added to the supernatant to 15% saturation, and precipitate was removed by centrifugation at 12,000g for 10 min at 4 °C. The supernatant was brought to 30% saturation with ammonium sulphate and the precipitate collected by centrifugation, dissolved in TEGDZ50 (50 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 10% glycerol, 5 mM DTT, 50  $\mu$ M ZnCl<sub>2</sub>, 50 mM NaCl), and dialysed against the same buffer. The T7X556-enriched fraction was applied to a phosphocellulose P-11 column equilibrated with TEGDZ50 and eluted with a 200–400 mM NaCl gradient. The pooled eluant was >90% pure T7X556 as assessed by silver staining after sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis. The peak fractions were pooled and dialysed against 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 75  $\mu$ M ZnCl<sub>2</sub>, 1 mM DTT, and applied to a Cibacron blue 3GA agarose column equilibrated with the same buffer. The column was washed with buffers containing 200 mM and 400 mM NaCl; T7X556 was eluted with buffer containing 800 mM and 400 mM NaCl. The protein was >95% pure as judged by silver-stained SDS gels and by amino acid composition analysis. Typical yields obtained were 1–2 mg of pure T7X556 per litre of starting culture. Purified T7X556 was dialysed against 50 mM citric acid pH 3.0, 10 mM EDTA, 10% glycerol, 1 mM DTT to remove zinc from the protein (see Table 1, line 2), giving apo-T7X556. Immediately before re-incubation with metal ions, the pH of the protein solution was raised to 7.9 by addition of 0.22 volume of 1.35 M Tris-HCl pH 8.8. After 30–60 min at 0 °C, DNA binding was assayed by gel mobility shift and DNase I footprinting.

We report here that a protein of relative molecular mass 19,000 ( $M_r$  = 19 K) encompassing the DNA-binding domain of the glucocorticoid receptor that has been overexpressed in *Escherichia coli* and purified to homogeneity reversibly ligates two Zn(II) or Cd(II) ions. We show that metal ions are required for specific DNA binding and proper folding. Using EXAFS (extended X-ray absorption fine structure) and visible light spectroscopies, we find that each Zn atom is coordinated in a tetrahedral arrangement by four cysteines.

A 150 amino-acid glucocorticoid receptor fragment that encompasses the DNA binding region (amino acids 407–556, denoted X556) was expressed in *E. coli* using the T7 system of Studier and Moffatt<sup>16</sup> and purified (see Fig. 1, legend) to virtual homogeneity. The purified protein (T7X556) specifically recognizes and protects the same GRE sequences as does the full-length receptor (Fig. 1a and data not shown). The protein was purified in the presence of Zn(II) and is found to contain approximately two Zn atoms per molecule, as assessed by atomic absorption spectroscopy (Table 1, line 1). Dialysis at low pH in the presence of chelating agents releases the Zn from the protein (Table 1), yielding an apo-protein that fails to bind to its target DNA (Fig. 1). DNA binding is restored, however, when apoT7X556 is preincubated with Zn(II) or Cd(II) (Fig. 1a); no other metal tested restored efficient DNA binding, although some weak binding is detected after addition of Co(II) (data not shown). Maximal DNA binding is achieved at 5  $\mu$ M Cd(II), whereas 250  $\mu$ M Zn(II) is required to elicit an equivalent effect (Fig. 1b). This result is consistent with our findings that T7X556 has an affinity for Cd(II) that is  $\sim 10^2$  times higher than for Zn(II) (B.F.L. and L.P.F., unpublished). At present it is not understood

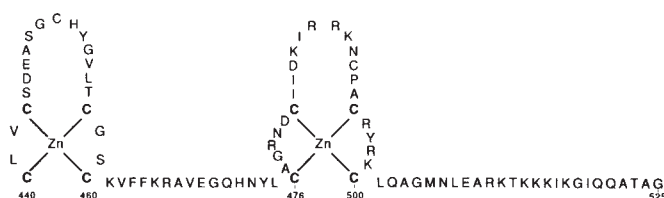




**Fig. 2** *a*, Zn K-edge XANES portion of the EXAFS spectra of T7X556 (solid line) and the model compound Zn(II) tetra-thiophenolate (dashed-dotted). Fluorescence intensity is plotted as a function of photoelectron energy in electron volts. *b*, The function  $k^3\chi(k)$  versus  $k$ , where  $k$  is the photoelectron momentum  $[(E - E_0)2m/\hbar^2]^{1/2}$  and  $\chi(k)$  is the X-ray absorption coefficient for T7X556 (top), and the model compound (bottom). *c*, The Fourier transform of the function in *b* for T7X556 (solid) and model compound (dashed-dotted). *d*, The back-transform of the function in *c* filtered with a 1.5- $\text{\AA}$  window to isolate the largest peak, corresponding to nearest-neighbour backscatter, for T7X556 (solid) and calculated model (dashed-dotted).

**Methods.** EXAFS data were collected at the Cornell High Synchrotron Source beam line C1. Monochromatic light was provided by the [220] Bragg planes of two silicon crystals arranged in series. The protein sample was prepared by gel-filtration on Sephadex G-25 (fine grain) equilibrated with 200 mM NaCl, 50 mM Tris-HCl pH 7.6; the buffer contained no thiol. The protein was concentrated to 2–3 mM with an Amicon Centricon 10 filter, and had a Zn:protein ratio of  $2.3 \pm 0.2$ . The solid model compound sample was diluted with lithium carbonate and ground to a fine powder in a glove box under a nitrogen atmosphere. Data from both the model compound and the protein were collected at room temperature in fluorescence mode with a Stern-Healds ionisation chamber<sup>24</sup> fitted with Soller vanes to reduce noise from scatter. Data from the model compound were also collected in transmission mode, and these spectra were indistinguishable from the data collected in fluorescence mode and also from transmission data reported previously<sup>25</sup>. The scanning step size was 2.0 eV. Both the protein and model compound data were normalized to their absorption edges using 9.760 keV as an initial estimate of  $E_0$ . The protein spectrum was smoothed using a three-point moving average, and the spectra were then converted to  $k$  space and multiplied by  $k^3$  to obtain amplitude functions with maxima near the centre of the  $k$  range used. Background was removed by fitting the data with a natural cubic spline. The background-corrected spectra ranged from 1 to 11  $\text{\AA}^{-1}$ ; beyond this range the signal to noise ratio fell below 2. The background-corrected spectra were Fourier-transformed to distance space (*c*) and a window 1.5  $\text{\AA}$  wide was used to isolate the first nearest neighbour backscattering peak. The isolated peaks of the protein and model compound were then back-transformed into  $k$  space where the values of  $r$  (the average distance of the first nearest-neighbours),  $A$  (their amplitudes), and  $\Delta\sigma^2$  (the difference in the square of their Debye-Waller factors), were compared. The protein spectrum could not be modelled using composite Zn(II) EDTA and Zn(SPh<sub>4</sub>)<sup>2-</sup> spectra, suggesting that neither N nor O are ligands in the protein. All data analyses were performed with software written by Stern and colleagues at the University of Washington.





**Fig. 3** Proposed coordination scheme for two zinc atoms within the DNA-binding domain of the glucocorticoid receptor. This model is consistent with phenotypes of mutant constructs, which demonstrate that changing cysteine residues predicted to be involved in metal coordination abolishes DNA binding as well as transcriptional regulation (ref. 26; P. Godowski and K.R.Y., manuscript in preparation). Note that coordination of two zinc atoms, each by four cysteines, would leave one cysteine unbound in each of the two 'fingers'. Consistent with this scheme, we find that spectrophotometric titration of the protein with para-hydroxymercuribenzoate<sup>27</sup> yields  $2.1 \pm 0.2$  reactive sulphhydryls per protein molecule. We have not shown definitively which four of the five cysteine residues in each finger are coordinating metal; therefore, other folding schemes are possible.

why DNA binding activity is reduced at elevated metal ion concentrations.

EXAFS spectroscopy was employed to establish the types and number of ligands involved in coordinating the Zn atoms and the distances between the ligands and the metal atoms. Data were collected from the protein and, in parallel, from Zn(II) tetrathiolophenolate ( $\text{Zn}(\text{SPh})_4^{-2}$ ; gift of Douglas Stephan, University of Windsor, Canada), a model compound shown by X-ray crystallography to contain a tetrahedral zinc:4-sulphur coordination complex<sup>17</sup>. Figure 2a compares the X-ray absorption near-edge structure (XANES) portion of the EXAFS spectra for the protein and for  $\text{Zn}(\text{SPh})_4^{-2}$ . In Fig. 2b, the EXAFS spectra have been background corrected and replotted in reciprocal-Å, and Fig. 2c compares the Fourier transforms of the two spectra; these data demonstrate a strong similarity in the protein and model compound EXAFS results. The dominant Fourier component arises from scattering between the Zn atom and its nearest neighbours; hence the correlation of the two spectra implies a corresponding similarity in the coordination number and the metal-ligand distances for the two specimens.

The nearest-neighbour peaks were filtered from the Fourier transform of the spectrum in Fig. 2c and then back-transformed into reciprocal-Å. We obtained nearest-neighbour coordination parameters of the Zn in the protein by varying coordination number, ligand-bond distance, and disorder parameter ( $\sigma^2$ ), using a least-squares method to fit the filtered, back-transformed model compound spectrum to that of the protein. The optimal fit (Fig. 2d) yields a coordination number of  $3.55 \pm 0.27$  for the protein with an average Zn-ligand distance of  $2.32 \pm 0.02$  Å, in close agreement with the  $2.353 \pm 0.016$  Å and  $2.30 \pm 0.02$  Å mean Zn-S distances observed in  $\text{Zn}(\text{SPh})_4^{-2}$  (ref. 17) and  $\text{TFIII}_A$  (ref. 18), respectively. The disorder parameters for the protein in solution and for crystalline  $\text{Zn}(\text{SPh})_4^{-2}$  are nearly the same ( $\Delta\sigma^2 = 0.001 \pm 0.002$ ), suggesting that the variation in Zn-ligand bond lengths of the two specimens is similar, and furthermore, that the two Zn sites of T7X556 are very close in detailed stereochemistry.

Two observations indicate that the ligating S atoms are arranged tetrahedrally about each Zn in the protein. First, the XANES of the protein and the tetrahedral model compound are similar (Fig. 2a). The XANES includes multiple scatter effects and in principle is a fingerprint for ligand symmetry. Second, the maxima in the optical absorption spectrum of T7X556 reconstituted with cobalt (which restores weak DNA binding when added to apo-protein) occur at 360 nm, which represents the Co-S charge transfer band, and at 734 nm and 688 nm, which are characteristic of d-d transitions of a high spin Co(II) centre in tetrahedral S complexes<sup>19-20</sup> (data not

shown). A possible coordination scheme for the two T7X556 fingers is shown in Fig. 3.

Apo-T7X556 reconstituted with Cd(II) shows a strong absorption band at 245 nm characteristic of Cd-S charge transfer<sup>17,21</sup>. The apo-protein titrates spectrophotometrically with a stoichiometry of 2.0 Cd(II) per molecule of protein (Table 1). The same stoichiometry was found by atomic absorption spectroscopy (Table 1, line 3). The crystal structure of Cd(II) tetrathiolophenolate, like the Zn complex, displays approximate tetrahedral coordination<sup>17</sup>, but with somewhat longer ligand bonds ( $2.535 \pm 0.013$  Å). Thus, the Zn(II)- and Cd(II)-reconstituted T7X556 differ in the diameter of their coordination spheres by 0.4 Å, and in S-S separation by 0.3 Å, although they both bind to GRE DNA (Fig. 1). Specific DNA binding *in vitro* therefore appears insensitive to the detailed stereochemistry of

**Table 1** Metal:protein stoichiometries

| Protein                          | Metal:protein   |
|----------------------------------|-----------------|
| T7X556*                          | $2.3 \pm 0.2$   |
| Apo-T7X556†                      | $0.02 \pm 0.01$ |
| Cd(II)-reconstituted apo-T7X556‡ | $2.1 \pm 0.2$   |
| Trypsin-digested T7X556 product* | $0.7 \pm 0.1$   |

Zn and Cd concentrations were determined using a Perkin-Elmer Model 306 atomic absorption spectrometer. Protein concentration was measured by amino acid analysis. Protein metal content was corrected for metal content of dialysate buffer.  $143 \pm 6$  μM T7X556 and  $184 \pm 16$  μM trypsin-digested protein have absorbances of 1.0 per cm path-length at 280 nm. The errors for the ratios tabulated reflect the spread in values of both the extinction coefficient and metal analysis.

\* Samples in 50 mM Tris-HCl, pH 7.6, 200 mM NaCl, 75 μM  $\text{ZnCl}_2$ .

† Samples in 50 mM citric acid pH 2.5, 10 mM EDTA, 1 mM DTT.

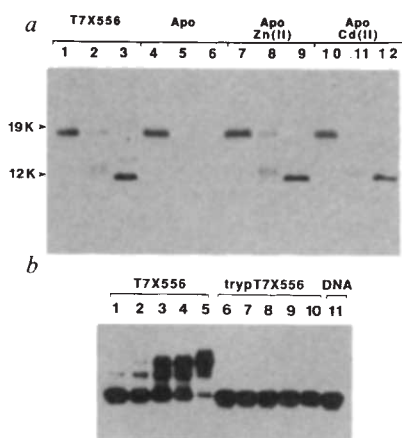
‡ Stoichiometry also determined by titration of apo-protein with Cd(II) and monitoring the 245 nm Cd-S charge transfer band. The value obtained by this method was  $2.0 \pm 0.1$  Cd:protein.

metal coordination, implying that metals do not confer specificity *per se*, but rather provide a scaffold for favourable DNA binding conformation.

We assessed the importance of metal ions in maintaining the structure of the receptor DNA binding domain by treating the metallo- and apo-proteins with trypsin. At room temperature, T7X556 yields a stable (>12 h) digestion product with an apparent  $M_r$  of 12 K, whereas the apo-protein is completely degraded under the same conditions in less than 10 min (Fig. 4a, lanes 1-6). Reconstitution of the apo-protein with Zn(II) or Cd(II) before trypsin treatment restores it to a form whose protease resistance is indistinguishable from the original, liganded protein (Fig. 4a, lanes 7-12).

The C-terminal cleavage sites in T7X556 reside within the second finger (see Fig. 4, legend). Consistent with these findings, the Zn:protein stoichiometry of the 12 K trypsin product is  $0.7 \pm 0.1$  (Table 1). Interestingly, the purified 12 K species does not bind to GRE DNA (Fig. 4b, lanes 6-10); nor does a linker-scanning mutant in the first finger (P.J. Godowski and K.R.Y., unpublished results). In addition, a mutation altering the basic residues downstream of the fingers (see Fig. 3) virtually abolishes DNA binding *in vitro* as well as a characteristic DNase hypersensitive site *in vivo* (P. J. Godowski, L.P.F., J. Vanderbilt and K.R.Y., unpublished results). Thus, both fingers appear necessary but perhaps insufficient for specific recognition of DNA by the glucocorticoid receptor.

Our results show that the DNA binding domain of the glucocorticoid receptor coordinates two metal ions, each through a tetrahedral arrangement of four cysteine sulphurs. Although cadmium appears to have a higher affinity for the protein than does zinc, we have recently found by inductively-coupled plasma mass spectrometry that purified native glucocorticoid receptor contains zinc, but very little cadmium (B.F.L. and L.P.F., unpublished results), inferring that zinc is the natural



**Fig. 4** Probing T7X556 structure and function by trypsin proteolysis. **a**, Metal binding protects against complete trypsin proteolysis. Samples were run on a 15% acrylamide SDS gel and silver-stained following trypsin digestion; control, T7X556 before trypsin addition (lane 1), and after 10 min (lane 2) and 8 h (lane 3) of digestion; apo-protein before (lane 4), and after 10 min (lane 5) and 8 h (lane 6) of digestion; Zn(II)-reconstituted apo-protein before (lane 7) and after 10 min (lane 8) and 8 h (lane 9) of digestion; Cd(II)-reconstituted apo-protein before (lane 10), and after 10 min (lane 11) and 8 h (lane 12) of digestion. All three of the most probable C-terminal trypsin sites (inferred from determinations of amino acid composition to be at residues 490, 496 and 498) reside within the second finger. In several experiments (data not shown), at least three discrete cleavage products have been observed after partial trypsin digestion, consistent with the proposed C-terminal cleavages within the second finger. These intermediates are then chased into a single 12K species. The N-terminal cleavage site (determined by sequencing the amino-terminus of the purified digestion product) is in an upstream nonreceptor linker sequence. **b**, Purified 12K trypsin fragment of T7X556 in the presence of metal ions does not bind GRE-containing DNA. Increasing amounts (4.75–75 ng) of intact T7X556 (lanes 1–5) or 12 K trypsin product (lanes 6–10) were mixed with 0.025 pmol of end-labelled 250 bp GRE DNA and assayed for DNA binding by gel retardation. Lane 11, input DNA.

**Methods.** One volume of intact, metal-stripped or metal reconstituted protein (prepared as in Fig. 1, except that 90  $\mu$ M ZnCl<sub>2</sub> or CdCl<sub>2</sub> was used for reconstitution) was mixed with 2.8 volumes of 44 mM Tris-HCl pH 8.23, 3.7 mM CaCl<sub>2</sub> and 1.2  $\mu$ g ml<sup>-1</sup> trypsin-TPCK at room temperature. Aliquots were removed during the time course, mixed with SDS sample buffer, and heated for 3 min in a boiling water bath. The trypsin-digested protein was prepared by treating 1 mg ml<sup>-1</sup> T7X556 in 34 mM Tris-HCl pH 8.23, 2.8 mM CaCl<sub>2</sub> and 0.9  $\mu$ g ml<sup>-1</sup> trypsin-TPCK at room temperature for 4–8 h. Benzamide was added to 2 mM and the sample was dialysed against 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 75  $\mu$ M ZnCl<sub>2</sub>, 1 mM benzamide and purified on a Cibracon blue column as described for intact T7X556 (Fig. 1). As with T7X556, the trypsin-digested protein eluted at 800 mM NaCl buffer. The purified protein was more than 95% pure on silver-stained gels. The apparent molecular weights of the undigested and digested proteins, determined with calibration standards on 15% acrylamide SDS gels are 20.8 K and 12.5 K respectively; molecular weights inferred from sequence are 18.8 K and 11.7 K, respectively.

coordinating metal. Proteolytic digestion of the metallo- and apo-protein forms of T7X556 indicates that metal coordination is essential for proper folding of the protein into its native, active structure. In this way the metal ion manifests itself as an essential cofactor for DNA binding. As the coordinating cysteines are conserved among the steroid receptors, the metal coordination scheme described here for the glucocorticoid receptor is probably common to all members of the steroid receptor family.

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**Letters to Nature** are short reports of outstanding novel findings whose implications are general and important enough to be of interest to those outside the field. Letters should not have more than 1,000 words of text or more than four display items and should not occupy more than two pages of *Nature*. The first paragraph should describe, in not more than 150 words, the origins and chief conclusions of the study. Letters should not have subheadings or more than 30 references.

**Commentary** articles deal with issues in, or arising from, research that are also of interest to readers outside research. Some are commissioned, most are unsolicited. They are normally between one and four pages of *Nature* in length.

**News and Views** articles are intended to inform non-specialist readers about a recently published advance. Suggestions should be made to the News and Views Editor. Illustrations are welcome. Proposals for meeting reports should be agreed in advance.

**Scientific Correspondence** is for the discussion of scientific matters, including contributions published in *Nature*. Priority is given to contributions of less than 500 words and five references. Figures are welcome.

**Submission.** All manuscripts may be submitted to either London or Washington. They should not be addressed to editors by name. Manuscripts or proofs sent by air courier to London should be declared as 'manuscripts' and 'value \$5' to prevent the imposition of import duty and value-added tax (VAT).

All contributions submitted for publication in *Nature* should conform with these rules.

**Manuscripts** should be typed, double-spaced, with a good-quality printer, on one side of the paper only. Four copies are required, each accompanied by lettered artwork. Four copies of half-tones should be included. Reference lists, figure legends and so on should be on separate sheets, all of which should be double-spaced and numbered. Copies of relevant manuscripts in press or submitted for publication elsewhere should be included, clearly marked as such. Revised and resubmitted manuscripts should also be clearly marked as such and labelled with their reference numbers.

**Titles** should say what the paper is about with the minimum of technical terminology and in fewer than 80 characters. Authors should avoid active verbs, numerical values, abbreviations and punctuation and should include one or two key words for indexing purposes.

**Artwork** should be marked individually and clearly with the author's name, figure number and, when known, manuscript reference number. Original artwork should be unlettered. Ideally, no figure should be larger than 28 by 22 cm. Figures with several parts are permitted only if the parts are closely related, either experimentally or logically. Suggestions for cover illustrations, with captions, are welcome. Original artwork (and one copy of the manuscript) will be returned when a manuscript cannot be published.

**Colour Artwork.** A charge of £500 a page is made for 4-colour figures. Inability to meet these costs will not prevent the publication of essential colour figures if the circumstances are explained.

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**References** should be numbered sequentially as they appear in the text, followed by those in tables and finally those in the figure legends. Reference numbers apply only to papers published or in the press, and a different number should be given to each paper cited. All other forms of reference (including unreferenced abstracts) should be included in the text as personal communication, manuscript in preparation or preprint (with number and institution where appropriate). Text should not be included in the reference list. References should be abbreviated according to the *World List of Scientific Periodicals* (fourth edition, Butterworth, London, 1963–65). The first and last page numbers should be cited. Reference to books should clearly indicate the publisher and the date and place of publication.

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**Footnotes** should not be used except for changed addresses or to identify the corresponding author if different from the first-named.

**Acknowledgements** should be brief. Grant numbers and contribution numbers are not allowed.



# Fetal bovine serum alternatives

D.W. Jayme, D.A. Epstein and D.R. Conrad

*With the current shortage in fetal bovine serum, researchers are turning to a variety of options for cell and tissue culture.*

FOR nearly a century, scientists have supplemented buffered salt solutions with various synthetic nutrients and humoral fluids to reproduce *in vitro* the physiological environment of tissues and organs<sup>1,2</sup>. Fetal bovine serum (FBS) is the most universally applicable cell culture additive for the stimulation of cellular proliferation and biological production.

Many factors influence the cost and availability of FBS. FBS is obtained as a by-product of the cattle industry, and its supply is subject to the same constraints and fluctuations. Cyclical patterns in herd sizes, beef and dairy prices, feed costs, weather conditions and dietary trends all influence the harvest volume of FBS. Furthermore, potential low-cost FBS from non-US sources is contaminated by exotic viruses, making it unsuitable for importation and laboratory use. The production of biological products using large-scale mammalian cell bioreactors has contributed to the shortage by placing increasing demands upon the finite supply of FBS.

Even if the supply of FBS were infinite and the cost were tolerable, there are other reasons to explore media alternatives. FBS is an undefined fluid which exhibits lot-to-lot variability in biochemical composition and culture performance (*Art to Science in Tissue Culture*, Vol. 5, 3-4, Hyclone Laboratories, 1986). Serum components can both stimulate and inhibit the desired cell culture response<sup>3</sup>, and serum-supplemented media may be unable to support growth of specific cell types or to prevent fibroblast overgrowth<sup>4</sup>. Bulk serum proteins may potentially reduce bioreactor yields, elicit artefactual responses and interfere with downstream purification in bioprocessing applications<sup>1,5-7</sup>. To overcome concerns regarding the availability, consistency, cost, and imprecise component definition of FBS, considerable effort has been expended to define culture conditions which support cell growth and function while reducing or eliminating the requirement for FBS<sup>1,7-9</sup>.

## Medium optimization

A superficial review of the cell culture media which are commercially available or reported in the literature reveals a broad diversity in composition and intended application. Different nutrient formulations preferentially support either cloning density or high-density requirements, function in open or closed atmospheric gas milieu, and demonstrate specificity for certain cell types or tissues<sup>2,7</sup>. In some

applications, it is best to use a "two-stage" culture system, where the supplemental nutrient composition can be modified to promote either population expansion or static culture production.

It is beneficial to optimize the exogenous culture environment before working with a novel cell type or initiating a new line of research. We urge investigators to invest the time to optimize the culture fluid, using more highly-enriched media to reduce or even eliminate the FBS requirement, rather than supplementing basal formulations with excessive FBS<sup>1</sup>.

The qualification of novel culture formulations should include multiple subcultures in the "test" medium and comparison of cell culture efficacy with FBS-supplemented reference medium. Many cell types can survive a single passage in sub-optimal medium through residual

**Table 1** Methods for reducing fetal bovine serum requirement

### Optimization of existing medium

- Modified nutrient composition
- FBS lot performance screening

### Alternative mammalian serum substitution

- Substitution by calf, newborn calf or horse sera
- Growth factor supplemented calf serum
- Serum fractions
- Volumetric serum blends

### Reduced serum media

- Growth factor enriched media
- Supplemental growth factor concentrates

### Defined media

- Serum-free media
- Protein-free media

stimulation from the previous culture fluid: maintenance subculturing dilutes such artefacts.

Qualification of a "new" culture environment should not focus exclusively on growth rate, but should also include analysis of culture density, cell viability, cell attachment and ability to elicit the target biological response. Most cell types demonstrate an adaptive "lag" phase in growth in response to an alteration of the nutrient composition, during which selective pressure enriches the culture for subpopulations exhibiting the preferential growth phenotype. Negative aspects of this selection may be minimized by altering inoculation density, and other adaptive "weaning" techniques<sup>10,11</sup>.

The FBS requirement can be reduced and the lot-to-lot variability minimized

through pre-screening of FBS lots for specialized applications. Several commercial suppliers perform sensitive assays to determine the suitability of individual FBS lots for customer applications based upon bioassay performance<sup>12</sup> and biochemical composition.

## Alternative sera

Expanding upon the quality enhancements pioneered in the collection and processing of FBS, serum suppliers have substantially improved the performance of alternative mammalian sera for various cell culture applications. We have examined multiple lots of alternative mammalian sera from various commercial sources using cell-based performance assays<sup>13</sup>. These studies demonstrated that sera derived from calf, newborn calf and donor horse sources may substitute for FBS without sacrificing proliferative rate, maximal cell density, cloning efficiency and biological yield in many anchorage-independent cell culture applications. In contrast, adherent cells exhibited specific preferences for individual sera: some cell types required FBS, while others performed best in a media supplemented with alternative sera. Generally, calf serum is the preferred substitute for FBS in adherent cell cultures.

Alternative mammalian serum is available in greater supply, and is more uniform between lots than FBS, permitting raw material cost reduction without loss of culture yield. Its disadvantages are that it contains altered compositions of certain hormones and other serum factors, and it has a high level of proteins (particularly immunoglobulins) which may complicate some culture applications.

Calf sera supplemented with defined factors, organ extracts or inorganic salts have demonstrated performance comparable to FBS for many adherent cell applications. Fractionated alternative sera have the additional advantages of lower gamma-globulin content and enhanced stability. Various laboratories have achieved substantial cost reductions without sacrificing performance by volumetric blending of FBS with other bovine sera<sup>14</sup>.

## Fortified media

In the technical summary of a recent bio-engineering conference, the conveners reported that "... the most economic way of reducing serum is the use of a low serum medium supplemented with other growth promoting factors, as opposed to

completely serum-substituted or even defined medium<sup>15</sup>. Growth factor-enriched media supplemented with low levels of FBS (or alternative serum) exhibit unique advantages by combining the economy of serum reduction with the broader range of cell culture uses supported by a serum-supplemented medium.

Several commercially available reduced serum media support proliferation of hybridomas and other suspension cultures at serum supplementation levels of less than 0.5 per cent<sup>16</sup>. Once the culture has been established at high density in a spinner flask, stirred tank reactor, airlift fermentor or hollow fibre unit, perfusion may be serum-free to facilitate product recovery and purification.

Reduced serum media have also been useful for monolayer cultures, where a primary contribution of serum is to facilitate cell attachment. Adherent cell lines may be maintained indefinitely in these growth factor-enriched media with a 60–80 per cent reduction in serum requirement<sup>14–16</sup>. Once cells have attached at the appropriate inoculation density, medium

replenishment may be serum-free to reduce cost and serum artefacts.

Some investigators prefer to retain the synthetic nutrient medium traditionally used in their laboratory, rather than switch to a novel, reduced serum medium. Such laboratories may still enjoy the benefits of serum reduction, by purchasing purified growth factors and designing a supplemental cocktail or by adding commercially available growth factor concentrates to their nutrient formulation.

### Defined media

The universality of culture medium efficacy appears to be inversely correlated with the degree of definition of its components<sup>1</sup>. Thus, while a serum-supplemented medium formulation may support the growth of many different cell types, serum-free media are generally more cell-specific<sup>2</sup>. There are a variety of serum-free media<sup>3</sup>, which may be suited for the culture of a specific cell line, but be unable to support the growth of a related line.

The use of totally defined, serum-free media may be advantageous in attempts to

culture functionally differentiated cells, in the investigation of regulatory phenomena, and for applications requiring component definition to facilitate downstream product purification or to satisfy Good Manufacturing Practices requirements<sup>1,3</sup>. However, due to the cost, scarcity and instability of some additives, serum-free media are not necessarily less expensive than other cultivation methods. Additionally, serum serves multiple functions in the *in vitro* environment, such as providing essential hormones, binding and attachment factors, pH buffering, protease inhibition, and binding and inactivation of toxic materials<sup>1,9</sup>. Thus, totally serum-free media must compensate not only for the nutritional contributions of FBS, but also for its ancillary functions.

Specialty media for defined applications are commercially available<sup>8</sup>, including serum-free and protein-free media for hybridoma and other suspension culture applications<sup>10</sup>. Defined formulations have also been designed for the growth of epithelial cells<sup>6</sup>, for virus<sup>1,5,14</sup> and enzyme<sup>1,8</sup> production, and for adoptive cancer immunotherapy<sup>17</sup>.

Multiple substitutes for FBS are available that provide significant benefits to the end user without sacrificing biological performance (Table 1). Effort should be directed — both at the laboratory and the commercial scale — towards the optimization of the nutrient medium with the goal of reducing or eliminating the culture requirement for FBS. □

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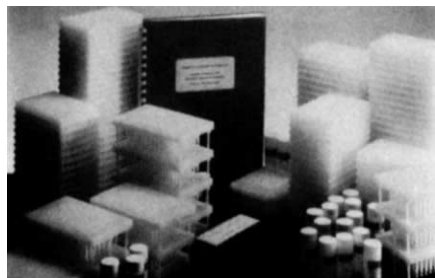


# Cell biologists convene in Canada

Nearly 100 exhibitors will be showing their wares at next week's 4th International Congress of Cell Biology in Montreal, Canada. Below are some selections from the booths, including media, cell lines, and microscopes.

BECKMAN Instruments is now distributing a **reference retrieval software package** produced by Research Information Systems (*Reader Service No. 101*). Called the Reference Manager, the program can search out and select a full reference from 20,000 entries in less than five seconds, says Beckman. The program stores up to 32,000 bibliographic references, and can format a manuscript to include references from a user's database. Users can add their own reference materials to the database by entering key words, abstracts, or total text, and by downloading references from online databases. Reference Manager is compatible with the Medline, BRS Colleague, BIOSIS and Dialog databases, and with the WordStar, WordPerfect and Microsoft Word word processing programs. The program runs on both IBM PC/XT/AT and PS/2 computers, and microcomputers which support MS/PC-DOS operating systems. Storage requirements are a hard disk with a memory capacity of 384 Kb RAM. Reference Manager costs \$630 (US), and is available from Beckman worldwide except in Australia, Austria, Belgium, France, Germany, Holland, Italy, Luxembourg, New Zealand and Switzerland. Beckman is in booths 212 and 218.

The concurrent synthesis and screening of hundreds of peptides is possible with the new **epitope scanning and mimotype design kits** from Cambridge Research Biochemicals, exhibiting in booth 324 (*Reader Service No. 102*). The kits are based on the multipin synthesis technique, which uses the Fmoc-solid phase synthesis chemistry to create peptides at the tips of 96 moulded polyethylene pins assembled into a plastic holder designed with the spacing of a microtitre plate. Once the peptides have been synthesized on the pin tips, they can be tested for their antibody binding capability by conventional ELISA procedures. Cambridge Research Bio-



Epitope scanning from Cambridge Research Biochemicals.

chemicals says in some cases the synthetic peptides have been used for up to 50 tests. The £2,500 (UK) epitope scanning kit contains all the necessary reagents for identifying the synthetic peptides homologous to the antigenic determinants, allowing the precise modelling of the antibody binding site. To take things one step further, the £4,250 (UK) mimotype design kit can be used to construct peptides which mimic, but are not actually homologous to, the epitopes of a particular antigen. Both kits are supplied with software which computes peptide sequences and synthesis schedules, and performs data analysis.

Medical Systems Corporation has announced the development of a new **pico-injector** for biomedical applications



Medical Systems Corp's way to microinject cells. at the cellular level (*Reader Service No. 103*). The \$2,495 (US) model PLI-100 pico-injector microinjects volumes ranging from microlitres to femtolitres through micropipettes. Medical Systems says reproducible delivery is achieved by the precise regulation of pressure and time, and the vacuum is initially supplied to fill the delivery pipette. Regulated balance pressure is applied between injections to prevent the initial dilution of the delivered material by capillary action. Medical Systems Corporation is in booth 601.

GIBCO Laboratories/Life Technologies, Inc. has a new serum-free medium for the **activation of lymphokine-activated killer (LAK) cells** in adoptive immunotherapy research and clinical studies (*Reader Service No. 104*). The AIM V medium is specially formulated to provide the optimal growth of human lymphocytes *ex vivo*, without the danger of viral contamination associated with serum. GIBCO says the AIM V medium is tested to ensure functional growth, low endotoxin levels, non-reactivity to hepatitis B surface antigen and human immunodeficiency virus type I antibody, and absence of microbial contamination. Each

lot is performance tested by a chromium release assay to confirm IL-2-dependent, cell-mediated lysis of target tumour cells. The AIM V medium does not require supplementation with undefined serum and has been proven to increase LAK cell activity in culture periods of up to seven days, according to GIBCO. GIBCO is exhibiting in booths 211, 213 and 215.

## Hybridoma hints

Human fibroblast cultures, when stimulated with interleukin-1 (IL-1), produce a growth factor for B-cell hybridoma and plasmacytoma cell lines named **interleukin-6 (IL-6)** (*Reader Service No. 105*). Janssen Biochimica processes crude IL-6 from MG-63 cells by sequential adsorption to controlled pore glass beads, antibody affinity chromatography and gel filtration to produce IL-6 which it says has a specific gravity of  $10^9$  units  $\text{mg}^{-1}$ . The purified 26-kD protein product has been variously referred to as interferon- $\beta_2$ , after a postulated fibroblast secretory protein with so far no unambiguously defined function, and B-cell stimulatory factor 2 for its T-cell product processing differentiation stimulatory effect on B-cell lines. Janssen Biochimica sells crude IL-6,



Interleukin-6: one of the lymphokines from Janssen.

highly purified IL-6 (free of IL-1) and recombinant IL-6. Janssen is exhibiting in booth 510.

Four new **immunoglobulin-producing hybridomas** are available from the American Type Culture Collection (*Reader Service No. 106*). HB8755 is a human lymphoblastoid cell line which produces monoclonal antibody to human T-cell leukaemia virus type I. HB8049, HB8612 and CRL1759 are mouse/mouse hybridomas which produce monoclonal antibody to the p27 protein of feline leukaemia virus, to normal human B cells and derived malignancies, and to the B chain of the plant toxin ricin, respectively. More information on other cell lines and hybridomas deposited with ATCC can be found in booth 302.

## Growth environments

Costar, exhibiting in booths 412 and 414, has announced the availability of its **Gel-Well cell culture chambers** for the culture of anchorage-independent cells such as cancer cells and lymphocytes (*Reader Service No. 107*). Gel-Wells consist of an inert, transparent, porous hydrogel



Costar's Gel-Well cell culture chambers.

matrix in the shape of a petri dish. Costar says the non-binding property of the hydrogel encourages the growth of cancer cells and specifically discourages the attachment and growth of normal cells. When Gel-Wells are placed in a tissue culture dish and equilibrated with growth medium, they form a two-compartment system that allows free diffusion of nutrients and waste products. The convex interior is designed to concentrate cells so that they have access to localized conditioning factors. Gel-Wells are available in a 15 mm in diameter size with a working volume of 0.2 ml, and in a 30 mm in diameter size which holds 1.0 ml. Gel-Wells can also be used for the small-scale production of secretory products, monoclonal antibody production, and co-cultivation of segregated cell populations.

Becton Dickinson's Microbiology Systems division has improved **prepared, plated media** for the isolation of *Legionella* species from clinical and nonclinical specimens (*Reader Service No. 108*). Working in collaboration with GIBCO Laboratories, Becton Dickinson has produced a new formulation of buffered charcoal yeast extract (BCYE) agar that exhibits higher fluorescence to aid in the recovery and isolation of *Legionella*. Becton Dickinson sells packages of ten



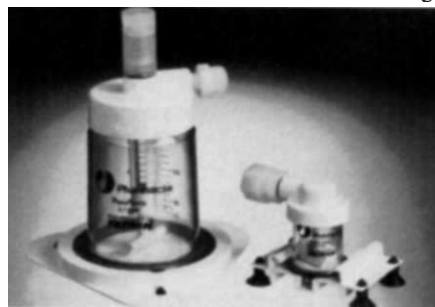
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agar plates of BCYE agar, BCYE differential agar, BCYE selective agar with colistin/cephalothin/vancomycin/cyclohexamide, BCYE selective agar with polymyxin B/anisomycin/cefamandole and BCYE selective agar with polymyxin B/anisomycin/vancomycin for \$9.50–15.50 (US). Becton Dickinson is exhibiting in booths 201 and 203.

## Concentration tips

Amicon has what it says is the first **microconcentrator tubes** capable of concentrating and desalting oligonucleotides, peptides, small proteins and growth factors (*Reader Service No. 109*). The Centricon-3 microconcentrator tubes, designed with a 3,000 MV cutoff, are the newest of Amicon's disposable centrifugal devices for sample preparation. Amicon says the Centricon-3 tubes provide sample recovery of over 90 per cent, performing concentrations of between 10- and 25-fold. The Centricon-3 consists of a concentrator and reservoir, filtrate cup, retentate cup and cap, and must be used with a fixed-angle rotor centrifuge capable of between 6,500 to 7,500  $\times g$  which accepts 17  $\times$  100 mm tubes. Amicon, exhibiting in booth 307, sells the Centricon-3 in packs of 24 for \$75 (US). Two other Centricon microconcentrators — the Centricon-10 and Centricon-30 — offer 10,000 and 30,000 MV cutoffs, respectively.

Pharmacia LKB Biotechnology now offers 10 ml and 150 ml **stirred cells for ultrafiltration**, for use in concentrating,



Pharmacia's ultrafiltration stirred cells come in 10 ml and 150 ml sizes.

dialysing or purifying small volume solutions and suspensions (*Reader Service No. 110*). Pharmacia LKB says the stirred cells are easy to use because they only have three components, the membrane is encapsulated to prevent handling, each cell has a colour-coded support base to identify its molecular weight cutoff and membrane type, and the cells are disposable, so they never have to be cleaned. Three different membrane chemistries are available: the Nova series has a polyether-sulfone (PES) membrane known for its strength, the Omega series has a modified PES membrane with low protein-binding properties, and the Alpha series has a modified PES membrane which maintains high flux in the presence of anti-foam

agents. Each series is available in nine different molecular weight limits, ranging from 3 kD to 1 M. Pharmacia LKB is in booths 219 and 221.

## The better to see . . .

Carl Zeiss, exhibiting in booths 500–506, has launched a new range of **inverted microscopes** named the Axiovert series (*Reader Service No. 111*). The Axiovert series of microscopes are based on a U-shaped stand with a three-point stage support for stability. Zeiss says all of the



The Axiovert from Carl Zeiss.

microscopes feature infinity colour-corrected system optics, long-distance objective and condenser systems to allow viewing of cultures within dishes of all sizes and wall thicknesses, and system integration, which incorporates all contrast-enhancing techniques without loss of image quality. Four models are offered in a number of configurations equipped for transmitted light examinations, reflected light observations, or reflected light fluorescence. All models have photo-documentation and television capabilities.

Wild Leitz has introduced a **fixed-stage tissue culture microscope** designed especially for micromanipulation studies (*Reader Service No. 112*). The Labovert's inverted stage is immobile and low-lying, allowing an unobscured view of the specimen and sufficient room for micromanipulation tools. Leitz says the Labovert can be converted easily from bright field to dark field, interference contrast and polarized light viewing, without disrupting microtool settings. Wild Leitz is exhibiting in booths 619 and 621.

## Setting it straight

The article on the separation of nucleic acids by HPLC which appeared in the product review section in the 7 July issue (*Nature* 334, 88; 1988) contained four errors in the reference list. References 1–3 and 5 are in the journal *Biochromatography*, not in the journal *Biochemistry*, as published. □

*These notes are compiled by Carol Ezzell from information provided by the manufacturers. To obtain further details about these products, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.*